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Biochemical Characterization of the Release of FAK Autoinhibition

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Biochemical Characterization of the Release of FAK Autoinhibition

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**Dissertation submitted to the
School of Medicine at West Virginia University
in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy
in
Biochemistry & Molecular Biology**

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conformation change**

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Abstract

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Focal adhesion kinase is an essential nonreceptor tyrosine kinase that plays an important role in development, in homeostasis and in the progression of human disease. Multiple stimuli activate FAK, which requires a change in structure from an autoinhibited to activated conformation. In the autoinhibited conformation the FERM domain associates with the catalytic domain of FAK and PI(4,5)P₂ binding to the FERM domain plays a role in the release of autoinhibition, activating the enzyme. This work aims to better characterize the residues involved in the release of the autoinhibitory conformation. The first aim was to characterize the effects of point mutations found in the COSMIC database. None of these mutations had an impact on the FAK autoinhibited conformation, thus classifying FAK as a kinase in which overexpression, and not mutation, is the mechanism by which its activity is increased in cancer. The second aim was to assess the role of FERM domain basic residues at the interface of the FERM and kinase domains in the autoinhibited conformation, R184, K190, and K191. While mutation of these residues does not cause a significant shift in FAK conformation, the results do suggest a minor role in binding to the membrane through PI(4,5)P₂ and interacting with the kinase domain to maintain the autoinhibited conformation. The third aim was to characterize residues on the catalytic domain that were modeled binding with the membrane *in silico*. Constructs with these residues mutated to alanine exhibited defects in phosphorylation and failed to completely rescue the phenotype associated with *fak*^{-/-} phenotype fibroblasts demonstrating the importance of these residues in FAK function. The catalytic domain of FAK exhibited PI(4,5)P₂ binding in vitro and binding activity was lost upon mutation of putative PI(4,5)P₂ binding site basic residues. Collectively, these studies further characterize the structural elements that aid in maintenance of both the open and autoinhibited conformations of FAK.

Dedication

I dedicate this thesis to my biggest distraction, Godric Danger Gibat.

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List of Abbreviations

FAK	Focal Adhesion Kinase
PI(4,5)P ₂	Phosphatidylinositol-4,5-bisphosphate
FERM	band 4.1, Ezrin, Radixin, Moesin domain
GST	glutathione-S-transferase
LUV	large unilamellar vesicle
LMV	large multilamellar vesicle
BODIPY	dipyrromethene boron difluoride
PC	phosphatidylcholine
PE	phosphatidylethanolamine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CFP	cyan fluorescent protein
YFP	yellow fluorescent protein
FRET	fluorescence resonance energy transfer
PH	pleckstrin homology
PTB	phosphotyrosine binding
SH ₂	Src homology 2
ACP	alternate cationic patches

Chapter 1: Literature Review

Introduction

Focal adhesion kinase (FAK - also known as Protein Tyrosine Kinase 2 [PTK2]) is an essential non-receptor tyrosine kinase. FAK localizes to focal adhesions (FA) and there plays a pivotal role in the signaling at these specific sites and beyond. As a kinase an important role for FAK is phosphorylation of downstream substrates to transmit cellular signals. FAK has an additional function as a scaffolding protein and playing an integral part in the assembly of signaling complexes. Both catalytic and scaffolding activities are critical for biological function. Multiple extracellular stimuli regulate the activation of FAK. FAK is activated through integrin signaling, but is also activated in response to stimulation of growth factor receptors, cytokine receptors, and mechanical stimulation.

Given the broad range of stimuli that activate this kinase, it is not surprising that it controls important and general cell phenotypes. Physiologically, expression of the FAK gene is essential as *fak*^{-/-} mice die during in the early stages of embryonic development. FAK is important for maintenance of healthy tissues and has roles in human pathology. The best example is the significant role that FAK plays in cancer, where its functions in regulating tumor growth and metastasis make it a key target for new cancer therapies.

Considerable structural information about FAK is available. NMR and/or crystal structures of the three major domains have been determined. These structures have provided important insight into the structural regulation of catalytic activity as well as insight in to the binding of various proteins to FAK, and its scaffolding function. This literature review will sum up the important research that has defined FAK's structure and function in both healthy and diseased states.

Biological and Physiological significance of FAK

FAK in Development

FAK is essential for multiple stages of development. FAK deficient mouse embryos do not develop past the embryonic day 8.5 despite normal implantation [1]. Recent studies indicate that FAK signaling is important prior to implantation as well. FAK signaling is involved in the maturation of cumulus-oocyte complexes prior to fertilization and plays a role in the maturation into a healthy embryo [2]. Once the egg is fertilized, FAK is necessary for early embryonic cleavage and the coordinated cell movements during embryonic morphogenesis known as epiboly [3,4]. In *X. laevis*, FAK is required for tension dependent cadherin adhesion complex formation, which is necessary for mass cell migration during gastrulation [5].

FAK is expressed throughout the embryo, but its expression is highest in the mesoderm. Unsurprisingly, lack of FAK expression leads to mesodermal defects. FAK deficient embryos do not properly develop a head fold nor neural tube nor heart tubes [1]. Through cell type specific knockouts, FAK's role in later developmental stages of various tissues has been determined. Mice embryos with endothelial specific FAK knockout die between embryonic days 9.5 and 11.5, indicating the importance of FAK in the development of the vasculature [6,7]. Normal vasculogenesis occurs in the absence of FAK, however embryonic angiogenesis fails resulting in lethality. There is disagreement regarding the cellular defect responsible for this lethal phenotype. Defects in proliferation and survival are reported in one knockout mouse line, while defects in spreading and migration are attributed to the phenotype in the other model [6,7].

Several studies show that FAK is important for cardiac development in addition to the development of the vasculature. In *Xenopus laevis*, FAK is essential for the development of a multichambered heart through enabling the proliferation of myocytes necessary for chamber outgrowth and facilitating the looping morphogenesis of the heart tube [8]. In mice, FAK is integral

for the proper alignment of the outflow tract and development of the aortic arch [9,10]. FAK is essential for proper cardiomyocyte migration and proper development of the septum between ventricles. FAK and a naturally occurring dominant negative variant which lacks catalytic activity, FAK related non-kinase (FRNK), function in coordination to regulate the different stages of cardiac development. Through transient expression, FRNK facilitates cell cycle withdrawal of cardiomyocytes allowing them to terminally differentiate [11]. A conditional knockout of FAK in neural crest cells also causes a cardiovascular defect, in addition to craniofacial malformations. This study shows that FAK is imperative for the differentiation and motility of neural crest cells and suggests that a defect in FAK signaling may be associated this congenital defects [12].

FAK also functions in the developing nervous system to control multiple processes including neuronal migration, morphogenesis and myelination. FAK signaling influences anterior-posterior cell fate in the developing neural plate [13]. In *Xenopus laevis*, phosphorylated FAK is localized to neuronal growth cones and supports axonal pathfinding [14]. The process of axonal pathfinding is aided through netrin signaling in which FAK plays a role. FAK is necessary for the correct formation of axonal pathways, as it is a negative regulator of axonal branching and synapse formation [15]. More recently the role of FAK in netrin mediated axonal guidance was confirmed utilizing shRNA in primary mouse neurons [16]. FAK potentially inhibits cofilin activity, thus regulating dendritic spine formation [17]. A combination of conditional oligodendrocyte-specific and inducible FAK-knockout mice were used to investigate the role of FAK in myelination. This study found that FAK is involved in the early stages of neurological development. FAK controls the efficiency and timing of CNS myelination during its initial stages by regulating the outgrowth and remodeling of oligodendrocyte process [18]. As the neural tube closes, there is a large burst of cell expansion which FAK is partly responsible for [19]. FAK in neuroepithelium triggers rapid growth by a mechanosensory response to elevated pressure from the increase in cerebral spinal fluid [20].

Additional organ systems are also impaired by loss of FAK expression. FAK is essential for the differentiation of osteogenic cells through a mechanosensory mechanism [21]. FAK signaling also controls proper nephron tubulogenesis in the kidney [22]. These studies show the role played by FAK in the development of multiple organ systems and that its signaling may impact multiple areas of the body.

FAK in the Maintenance of Tissue

Past fetal development, FAK is important for the remodeling and maintenance of adult tissues. FAK activity is vital for proper wound healing. Keratinocyte specific FAK knockout show decreased healing. Furthermore, intestinal epithelial conditional FAK knockout mice show FAK's importance in maintaining an intact mucosa [23,24]. FAK is also necessary for stress related responses prior to ischemia and healing after the injury. FAK myocyte knockout mice lack the cardioprotective signaling triggered by ischemic preconditioning [25]. A kidney specific FAK knockout model using an ischemia/reperfusion injury model shows that FAK is required for renal tissue wound healing [26].

Beyond wound healing, adult tissues also need to remodel in response to changing conditions. FAK continues to be important in this scenario as well. Inducible endothelial cell FAK knockout shows that FAK plays a role in the maintenance of blood brain barrier integrity [27]. A different endothelial FAK knockout model identified its importance in angiogenesis. However, the same study also showed that the related protein Pyk2 can compensate in some scenarios [28]. Mammary epithelial specific FAK knockout leads to insufficient mammary gland development during pregnancy [29]. FAK also plays a role in several stages of spermatogenesis as well as maintenance of the blood testes barrier [30,31]. Overall, these studies show that FAK expression and activity are essential to the proper function and maintenance of many tissue types in an adult organism.

FAK in Cellular Function

FAK has three major functions in the cell: controlling survival, proliferation, and motility. FAK contributes to survival signaling through a variety of pathways. Integrin signaling is imperative for a cell to sense it is adherent and in the correct location relative to other cells in a tissue, and should avoid a specific form of apoptosis, known as anoikis. FAK transmits integrin signals necessary for prevention of anoikis through a number of different signaling pathways [32,33]. Integrin signaling also promotes cell cycle progression in response to mechanical stimulation. FAK's mechanosensory role helps to regulate adhesion responsive proliferation signals [34].

Interactions with growth factor receptors by FAK also encourage survival, proliferation and motility through a variety of mechanisms. IGF1R can interact with and activate FAK leading to antiapoptotic signaling [35]. FAK activation in response to EGF and PDGF induces cell proliferation and migration [36]. Additionally, the HGF (scatter factor) receptor Met interacts with FAK directly through phosphotyrosines and leads to increased cell invasion [37–39]. It seems that FAK is a hub at which multiple growth factor receptor signals can be received and disseminated.

FAK also plays an integral role in the regular function of the cytoskeleton. It controls turnover at focal adhesions through interactions with GAPs and GEFs involved in the regulation of Rac and Rho [40]. FAK associates with 2 GAPs; GTPase regulator associated with FAK (GRAF) and PH and SH₃ domain containing Rho GAP (PSGAP); as well as two GEFs; RD2RhoGEF and p190RhoGEF, that all regulate the activity of Rho [41–44]. FAK also regulates cytoskeletal remodeling and actin polymerization through interactions with actin binding proteins Arp2/3 and N-WASP [45,46]. This control of actin, Rho and focal adhesion turnover rate lead to FAK's integral role in cell motility [47]. Forming new adhesions and dissolving old ones are key steps cell migration, and remodeling the cytoskeleton to reposition the cell mass over these adhesions is

necessary as well. In addition to coordination of adhesions and the actin cytoskeleton, FAK can play a role in directional persistence and mechanosensing [40,48].

Studies out of the Lim lab suggest an additional FAK function. In experiments reexpressing the N terminal portion of FAK they observed nuclear localization [49,50]. Follow up studies with full length FAK showed similar results [50]. This localization can impair the transcription of certain genes through interaction with the N-terminal transactivation domain of p53 [51,52]. Furthermore, FAK influences the overall structure of chromatin through binding of Methyl-CpG Binding Domain Protein 2 (MBD2) and inhibiting the action of MBD2 as a transcriptional regulator [53]. These observations may explain the effects of FAK on gene regulation and expression to promote survival and proliferation.

FAK in Human Disease

Cancer

Studies examining the role v-src oncogene and its tyrosine kinase in oncogenic transformation of fibroblasts led to the discovery of FAK as a substrate for v-src [54]. FAK was also independently isolated as a tyrosine kinase expressed in breast carcinoma [55]. While two other groups also independently identified FAK in other contexts [56,57], the two discoveries in the context of cancer suggested a potential role in oncogenesis and many studies have now confirmed the role of FAK in the development and metastatic spread of cancer. This conclusion is based upon three major observations: 1) FAK is overexpressed in many cancers, 2) aberrant FAK expression in some cancers is associated with poor prognosis, and 3) perturbation of FAK in a number of animal models blocks tumorigenesis and metastasis.

Initial studies to examine FAK in human cancer identified elevated FAK expression at the protein or RNA level in a variety of different tumor types. Interestingly, in melanoma elevated phosphorylation of FAK without overexpression was observed [58]. In a number of tumors, FAK

expression was elevated in advanced metastatic disease, and in melanoma elevated phosphorylation was observed in metastatic but not primary tumors. These findings originally suggested that FAK might be associated with advanced disease. However, other studies have demonstrated that elevated FAK expression in early stages of cancer and thus is not strictly associated with advanced stages of cancer. In some types of cancer, FAK overexpression is prognostic. For example, FAK overexpression in ovarian cancer and esophageal squamous cell carcinoma is associated with poor outcome [59,60]. In breast cancer, FAK overexpression has not been linked to prognosis but, amplification of the FAK gene is a poor prognostic indicator [61]. Not all studies have demonstrated a link between FAK expression or phosphorylation and disease outcome. Further analysis of some of these cancers might be warranted since the linkage of FAK expression to outcome may be more complex. For example, FAK overexpression is not prognostic in laryngeal cancer and neither is loss of E-cadherin expression. However, if FAK expression and E-cadherin expression are considered together they do serve as prognostic indicators. Patients with tumors exhibiting elevated FAK expression and low E-cadherin expression exhibit significantly reduced survival [62]. These examples demonstrate FAK overexpression, amplification, or phosphorylation are important in many tumor types and in some, but not all cases, this is indicative of poor prognosis.

Many experimental models have been employed to evaluate the role of FAK in tumorigenesis and metastasis. Anti-sense, dominant negative and RNA interference strategies have been employed to investigate the role of FAK in xenograph and orthotopic tumor models and an experimental model of metastasis. The results of these studies have implicated FAK in regulating the tumorigenic and metastatic properties of both murine and human cell line models of cancer, like in breast cancer models [63,64] and ovarian cancer models [65]. Since the FAK knockout is embryonic lethal, conditional knock out approaches were used to evaluate the role of FAK in additional animal models of cancer. In a chemical carcinogenesis model of skin cancer, FAK was

implicated both in the formation of papilloma and progression to carcinoma [66]. In transgenic models of breast cancer, multiple groups have demonstrated a role for FAK in tumor formation and metastasis [61,67,68]. These studies establish an active role for FAK in controlling tumor formation and metastasis in animal models.

Cardiac Disorders

While FAK is involved in several steps in the development of cardiovascular and may play a role in congenital defects, it also is implicated in cardiac disorders developed later in life. FAK is highly expressed in cardiomyocytes and is activated by both mechanostimulation, as well as angiotensin II and endothelin [69]. It is expressed in register with myofilaments near the sarcomeric A Bands but under stress FAK translocated to the Z discs of cardiomyocytes [70]. Because of its unique localization and activation in cardiomyocytes several groups have looked in to FAK's role in cardiac remodeling. In cell culture FAK is necessary for hypertrophic responses [71,72]. Cardiomyocyte FAK knockout mice showed that FAK is necessary for cardiac hypertrophy in response to stress [73]. It is interesting to note that a later study corroborated the need for FAK in stress response, but showed that it is not necessary for cardiomyocyte maintenance [74].

While some hypertrophic growth is a necessary stress response, FAK has been implicated in the hypertrophic maladaptation that leads to diastolic heart failure [75]. In comparisons of hearts from patients an increase in FAK expression and phosphorylation was detected in hearts with diastolic heart failure [76]. Mouse models agree with these findings, and siRNA knockdown of FAK can partially rescue heart failure [77]. Additional studies utilizing a cardiomyocyte specific transgenic strategy, suggest that FAK controls hypertrophic growth by activating both the AKT and mTOR pathways [78]. The mechanism by which FAK is regulated in cardiomyocytes and in hypertrophic growth signaling is unclear. The interaction of sarcomeric myosin with the FAK

FERM domain was established by small-angle x-ray scattering and this interaction negatively influences hypertrophy [79].

FAK is also expressed in the vascular endothelium as well as vascular smooth muscle cells. The response of FAK to mechanical stimulation as well as to growth factor signaling implicates it in hypertension and atherosclerosis. Shear stress in the vasculature can activate endothelial cell FAK [80]. VEGF stimulation of FAK enables endothelial remodeling [81]. Additionally, FAK is needed for vascular smooth muscle cell repositioning in the eutrophic remodeling response to hypertension [82]. Along with changes in the vasculature, FAK plays a role in the signaling and immune response at atherosclerotic plaques. Endothelial FAK/RSK signaling in response to oxidized LDL is needed for leukocyte recruitment and early atherosclerosis [83]. FAK knockdown mice show reduced endothelial cell permeability, suggesting that FAK activation could also increase the leukocyte population at atherosclerotic plaques. Macrophage response to plaques is a necessary step in atherosclerotic progression and FAK signaling is necessary for macrophage motility [84]. These studies show that FAK could be a potential target in the treatment of atherosclerosis.

The Other Family Member: Pyk2

There is one other member of the FAK family. Protein Tyrosine Kinase 2 (Pyk2) is structurally very similar to FAK, however its role in the cell is very different. Pyk2's nucleotide sequence is 46% identical to FAK, its amino acid sequence is 65% similar and exhibits the same domain organization and the structural characteristics as FAK. Like FAK, the Pyk2 gene also encodes an inhibitory splice variant [85].

Pyk2 is activated in response to growth factors and cytokines. Pyk2 is activated by an increase in cytoplasmic Ca^{2+} from stimulation by a ligand such as vasopressin or PDGF that triggers the Phospholipase C pathway [86]. While pyk 2 has a generally cytosolic localization, it Pyk2 plays

a role in regulation of the cytoskeleton during cell repulsion, as well as cell attachment and spreading in osteoprogenitor cells [87,88].

Pyk2 has a significantly different role in development as compared to FAK. Pyk2 is more restricted in its expression and function during development and Pyk2 $-/-$ mice are fertile and exhibit no visible malformations [89]. However, these mice do exhibit some deficits in the immune system. The Pyk2 $-/-$ mice have a reduced number of Marginal Zone B cells (found in the marginal zone of the spleen) and mount a defective humoral immune response [89]. Pyk2 null CD8 positive T cells are not properly activated in response to T cell receptor and LFA1 co-stimulation [90]. The macrophages and osteoclasts in Pyk2 $-/-$ mice are also defective. The macrophages show reduced infiltration toward inflammatory stimuli in vivo and exhibit polarization, contractility and motility defects in vitro [91]. Pyk2 $-/-$ osteoclasts produce an abnormal sealing zone and a defect in bone resorption, leading to osteoporosis [92]. Interestingly, a second group attributes the increased bone mass in Pyk2 $-/-$ mice to increased osteoblastogenesis and osteoblast activity, rather than differences in osteoclast activity [93]. Finally Pyk2 $-/-$ mice are also more prone to obesity and insulin resistance [94]. Overall, while FAK and Pyk2 are structurally very similar their functions do not significantly overlap. Pyk2 does pose an interesting target in understanding osteogenic diseases but with FAK playing a role in cancer and cardiac diseases, the two of the biggest factors in American mortality, it is the more significant research topic.

FAK Structure: A Multidomain Puzzle

The key to understanding the function and regulation of any protein is understanding its structure and how interacting proteins and ligands interact with that structure (Figure 1). The identification of domains within FAK was originally made based upon sequence homology and deletion analysis to identify functional regions of the protein. The general structure of FAK is very simple. There are four domains arranged in a linear fashion. The N terminus consists of the band

4.1, Ezrin, Radixin, Moesin (FERM) domain followed by the Kinase domain. These two domains are separated from the C terminal focal adhesion targeting (FAT) domain by an unstructured proline rich region [103].

FAK becomes phosphorylated at several sites [104,105]. Tyr 397 lies within the linker region between the FERM and kinase domains, and it is the major autophosphorylation site [106]. This site is also the site necessary for Src binding [105]. Tyr 577, 576, 861 and 925 are also key residues that are phosphorylated by Src. Tyr 576 and 577 are in the activation loop of the kinase domain and phosphorylation promotes maximal catalytic activity. In addition to tyrosine phosphorylation, several serine residues are phosphorylated in FAK. Phosphorylation of Ser 722 is regulated by GSK3 and the PP1 phosphatase and has an inhibitory effect on FAK catalytic activity [107]. Ser 732 is phosphorylated by Cdk5 and is essential for actin cytoskeletal reorganization, neuronal migration and centrosome function in mitosis and during neuronal migration [108–110]. Thus serine phosphorylation of FAK is as physiologically significant as tyrosine phosphorylation, and both types of phosphorylation can regulate FAK catalytic activity and scaffolding functions.

FAK Related Non Kinase (FRNK), is a naturally occurring splice variant of FAK consisting of solely the C-terminal portion consisting of the proline rich region and FAT domain. FRNK functions as a dominant negative mutant to block FAK function [111–113]. The FRNK Transcript is initiated from a second promoter within the FAK gene [114]. FRNK consists of the C terminal proline-rich and FAT domains of FAK and is missing the FERM and Kinase domains. The dominant negative function is through competition with FAK for localization to focal adhesions and possibly by titrating away FAK binding partners important for function [115]. FRNK has frequently been used as an experimental tool to evaluate the role of FAK in controlling biological function, but also is a physiological regulator of FAK, for example during cardiac development [11].

FERM Domain

Band 4.1, ezrin, radixin, moesin (FERM) domains are tri-lobed, typically N terminal domains [116]. They are commonly found in proteins that bind cytoplasmic regions of transmembrane proteins and often act as linkers between the cytoskeleton and plasma membrane. In addition to intermolecular interactions, FERM domains frequently mediate intramolecular interactions. The ezrin, radixin and moesin FERM domains each bind their C-terminal domains [117]. Amino acid sequence analysis showed both FAK and Pyk2 have N-terminal FERM domains, though the FAK family FERM domains share only 12-15% identity with the sequences of other FERM domains [118]. Crystal structures of the FAK FERM domain alone, and in complex with the catalytic domain are solved and provide considerable insight into FAK regulation and function [119,120].

Like all FERM domains, the FAK FERM domain has three lobes, the F₁, F₂, and F₃ subdomains. The F₁ subdomain spans residues 33 – 127 and consists of a five strand β sheet capped by an α helix. This subdomain exhibits an ubiquitin like fold. It has several structural features that differ from the F₁ subdomains of other FERM domains. The second α helix is longer than in most FERM domains, and the $\beta_1 - \beta_2$ and $\alpha_2 - \beta_5$ loop regions are also longer. In FAK, these loops contain 3₁₀ helices, a feature unique among the FERM domain containing proteins [119]. Each of these features are on the surface of the domain where structural differences could impact interactions with other proteins. The F₂ subdomain spans residues 128-253. The structure is entirely α helical, containing 4-core helices. This helical core is similar to that found in the acyl-CoA-binding protein [119]. There are several unique features of the F₂ subdomain. It has three segments containing 3₁₀ helices and a nine residue α_2' helix located between the α_2 and α_3 regions. This region of the protein forms a surface that docks with the catalytic domain. The F₂ subdomain also contains a basic patch on the apex of the domain that plays a significant role in FAK regulation. This patch contains the sequence ²¹⁶KAKTLRK²²² that is important for phosphatidylinositol and phosphotyrosine binding [121,122]. As they play a role in contact with the catalytic domain and

ligand binding, these two features mark the F2 subdomain as a key regulator of autoinhibition, unique among FERM domains. The final subdomain covering residues 254 to 352 is the F3 subdomain. It is a β sandwich capped with a C terminal α helix. This structure resembles a PH (pleckstrin homology domain), a PTB (phosphotyrosine binding) domain and an EVH1 (Ena/VASP Homology) domain. This structural similarity suggest a role in protein binding. However, the surface features are different on the surface of the FERM domain. So the F3 subdomain of FAK lacks the structural features of PH domains required to bind acidic phospholipids and the features of PTB domains required to bind tyrosine phosphorylated peptides.

Taken individually the subdomains of the FAK FERM domain are quite similar to those from other FERM containing proteins. The orientation of the individual subdomains in relation to each other is a little bit different. The ERM proteins, such as radixin, have a very symmetrical arrangement of the three domains leading most ERM family members to structurally overlap each other with a root mean square of less than 1 Å. FAK FERM aligns with radixin with a root mean square around 1.5 Å, due to a shift of the domains in relation to each other. The most noticeable shift is in the orientation of the F3 subdomain. It is rotated in such a way that the α helix is closer to the F1 subunit than in other FERM domains. The FERM domains of the ERM proteins have a phosphatidylinositol binding site in a shallow basic cleft between F1 and F3. In the FAK FERM domain the sequences between the F1 and F3 domains are not basic, the cleft between the two domains is narrower and it is occupied by part of the linker between the FERM and catalytic domains, thus precluding PI binding via this mechanism. What is especially interesting about the FAK FERM domain is that while the potential for PI binding is occluded in the F1/F3 cleft, a basic ridge on the F2 subdomain does bind to PIP2 [123].

The Linker

The linker between the FERM and kinase domains is an unstructured region spanning residues 352 to 415 and contributes to the structural distinctiveness of FAK FERM domain. The portion of the linker proximal to the FERM domain forms an antiparallel β sheet that binds on a groove on the F₃ subdomain. The linker, in this position completely buries the site analogous to the phosphatidyl inositol binding site on other F₃ subdomains. Two important features of the linker are Tyrosine 397, which is the major autophosphorylation site and Src SH₂ domain binding site and the nearby “PxxP” motif that acts as a binding site for the SH₃ domain of Src [104].

Kinase Domain

The kinase domain of FAK has been crystallized in three states: with the activation loop fully phosphorylated and bound to a non-hydrolysable ATP analog (fully activated conformation) (PDB 2JOL), alone with an unphosphorylated activation loop (inactive conformation) (PDB 1MQB), and unphosphorylated and bound to the FERM domain (autoinhibited conformation) (PDB 2JOJ, 2JOK) [120,124]. These structures demonstrate that like the catalytic domains of other protein kinases, the FAK Kinase is bilobed, with the N-terminal lobe containing a single α helix (the C α helix) and a 5 stranded β sheet, and the larger C terminal lobe that is mostly α helical. In FAK, the C α helix in the N lobe is rotated away from the C terminal lobe. The C α helix is in the same position in the active and inactive conformations, indicating that C-helix displacement is not a method of regulation of FAK [120]. In the unphosphorylated structures the activation loop is disordered. In the phosphorylated structure the activation loop adopts the β hairpin loop conformation seen in many other active kinases. In the unphosphorylated kinase structure with no ATP analogue present, the ATP binding site was a network of ordered water molecules, indicating a large amount of hydrogen bonding in the active site. The hydrophobic

pocket that contains the adenine base on ATP is bordered by M499. Additionally, ATP binding has been shown to create a small conformational change in the kinase domain, this could play a role in the allosteric regulation of FAK [125]. One structure of the unphosphorylated kinase domain contains a disulfide bond in the N lobe between cysteines 456 and 459 [124]. The cysteines are conserved in vertebrate FAKs and a regulatory role for this bond was suggested, however, other crystal structures do not contain this disulfide bond.

The crystal structure of the PYK2 kinase domain is very similar to that of the FAK kinase domain [126]. The ATP binding pocket of the PYK2 kinase domain cradles adenine with residues 431, 455, 487, 504, and 556, and the interaction is stabilized by interactions between the amino group and the N₁ nitrogen with residues 503 and 505.

Proline-Rich Region

The Proline-rich region is an unstructured flexible region spanning residues 687 to 918. While unstructured, this sequence contains several key scaffolding sites. First there are four “PxxP” motifs, two that are well defined, PR₁ (⁷¹²PPKP⁷¹⁵) and PR₂ (⁸⁷²PPKKPP⁸⁷⁷), and two that were recently identified in a study looking at cortactin interactions with FAK, PR₃ (⁷¹⁸PGYP⁷²¹) and PR₄ (⁸⁷⁹PGAP⁸⁷⁷) [127]. These motifs serve as binding sites for the SH₃ domains of a number of proteins. This study showed that interaction at PR₃ with cortactin is necessary for FAK activation in response to *Helicobacter pylori* infection [127]. The SH₃ domain of Crk-associated substrate (p130Cas) interacts with FAK at PR₁, and this interaction is vital for proper regulation of FAK mediated cell migration [128–130]. PR₂ interacts with the SH₃ domain of endophilin A2 and this interaction promotes Src-dependent phosphorylation of endophilin-A2 and reduced endophilin-A2 dependent endocytosis of MT1-MMP, leading to increased cell invasiveness [131]. GTPase regulator associated with FAK (GRAF) also binds to FAK via SH₃-mediated binding to PR₂, and this interaction is important for many Rho-based signaling events [41]. Other GAPs and GEFs including

p190RasGAP, Rgnef, and ASAP1 interact with PR1 and PR2. The PR motifs serve a scaffolding function, the recruit multiple proteins, many of which regulate actin polymerization and FA formation, thus these regions of FAK serve a critical role in the control of motility [132].

Phosphorylation sites are also key binding sites, especially for SH2 domain containing proteins. The proline-rich region of FAK contains an abundance of serine phosphorylation sites, as well as one threonine and one tyrosine phosphorylation site [133,134]. The functions of some of these in scaffolding and signaling have been identified, while the role of others has yet to be determined. Serine phosphorylation of the proline-rich region plays a significant role in the control of cell cycle and mitosis by FAK [133]. Phosphorylation on Serine 722 is regulated by GSK3 and PP1 and phosphorylation at this site increases cell spreading [107]. Serine 732 is phosphorylated by CDK5 [109]. This phosphorylation is linked to FAK localizing with the centrosome, microtubule fork and cytoplasmic dynein [110]. This localization may lead to microtubule arrangement, endothelial cell proliferation, and angiogenesis [108,135]. Tyrosine 861 has been long held as one of the Src phosphorylation sites on FAK, though a less prominent one than those in the kinase activation loop [136]. Phosphorylation of this residue has been linked to VEGF stimulated anti-apoptotic activity and cell migration [137]. Recently though, its role in FAK activity has been redefined. Using a modified Boyden chamber assay Chatterji et al, identified cells with increased migration in two different prostate cancer cell lines [138]. These more motile cells had increased Tyrosine 861 phosphorylation. An investigation of src family kinases (SFKs) in these motile subclones lead to the conclusion that yes kinase expression was increased and that yes preferentially phosphorylates tyrosine 861 [138]. Elevated yes expression and increased phosphorylation of FAK at tyrosine 861 were also observed in metastatic tumors in human patient samples [138]. There is much to be discovered regarding the roles of the phosphorylation sites in the proline rich region in assembling protein complexes.

FAT Domain

The FAT domain is the very C-terminal domain, and is responsible for FAK's localization to focal adhesions [139]. The efforts of a number of groups have yielded both crystal structures and NMR solution structures of the FAT domain [140–143]. The domain contains four amphipathic alpha helices that assemble into an antiparallel four helix bundle with an up-down-up-down right-handed topology. The crystal structure of the highly conserved FAT domain of Pyk2 has also been solved and this domain adopts a similar fold [144]. There is similarity in the FAT domain structure and the structures of the C-terminal vinculin tail, alpha-catenin and apolipoprotein 3, since all contain a right-handed antiparallel four helix bundles [140].

The most noticeable features of the FAT domain of FAK are two hydrophobic patches on the surface of the domain flanked by basic residues. One of these is at the interface of alpha-helices 1 and 4, and the other is on the opposite side of the molecule at the interface of alpha-helices 2 and 3. These hydrophobic patches were originally proposed as binding sites for FAK associated proteins, which has been confirmed by crystallographic and NMR studies [103,145,146]. The two hydrophobic patches of the FAT domain engage LD motifs of paxillin. The LD motif is a peptide motif repeated multiple times in the N-terminal half of paxillin and the paxillin related proteins, leupaxin and hic-5 [147,148]. The second and fourth LD motifs of paxillin function as FAK binding sites and each can form an amphipathic alpha-helix. The hydrophobic surface of these LD motifs docks with the hydrophobic patches of the FAT domain with acidic residues in the LD motifs interacting with the basic residues surrounding the hydrophobic patches on the FAT domain. While the conserved nature of the LD motifs and hydrophobic patches suggest interchangeable interactions, the docking of a peptide mimicking the LD2 motif to each binding site on the FAT domain exhibits different thermodynamic properties [103]. Several experimental approaches demonstrate a preference of the LD4 motif for the hydrophobic patch formed at the interface of alpha-helices 2 and 3 in the FAT

domain [146,149]. Similar interactions facilitate binding of LD motifs to the FAT domain of Pyk2 and the FAT homology domain of GIt1 [150].

Tyrosine 925 in FAK and tyrosine 881 in Pyk2 are sites of phosphorylation and mutation of these residues dramatically impairs recruitment of Grb2, a scaffolding protein that is important for FAK signaling [151,152]. These tyrosine residues reside within alpha helix 1 of the FAT domain and this structure makes them poor substrates for kinases and poor recognition motifs for the Grb2 SH2 domain, which both recognize tyrosines in an extended peptide conformation and not an alpha helix [153]. Thus for efficient phosphorylation and Grb2 binding, a conformation change in the FAT domain was proposed. One crystal structure of the FAK FAT domain revealed a domain swapped dimer, where alpha helix 1 of two different molecules were exchanged to form a dimer [141]. While this dimerization is not physiological, the result suggests conformational dynamics of the FAT domain during expression, purification and crystallization. Hydrogen exchange experiments support the hypothesis that the FAT domain is a dynamic structure and discrete molecular dynamics analysis of the FAT domain structure, guided by the results of hydrogen exchange, further support the model that alpha helix 1 can be displaced to facilitate transition from a helical to extended conformation of the peptide sequence containing tyrosine 925 [154,155]. These studies suggest that the FAT domain is dynamic, capable of adopting the four helix bundle and an altered conformation allowing phosphorylation of tyrosine 925 and Grb2 binding. Binding of the paxillin LD motifs to the FAT domain, particularly to the interface of alpha helices 1 and 4, stabilizes the four helix bundles and impairs tyrosine phosphorylation of these sites and Grb2 binding. Thus paxillin binding might regulate FAK signaling by regulating FAT domain structure, promoting specific signaling events while inhibiting others.

Autoinhibition and the Activation of FAK

The FERM domain was proposed as a negative regulatory element of FAK as deletion of the FERM domain resulted in elevated catalytic activity and constitutive tyrosine phosphorylation in cells [156–159]. Ezrin, radixin, and moesin all contain N-terminal FERM domains that regulate their activity by creating contacts with and folding over the active sites of more C-terminal domains [160]. Based upon autoinhibitory models of other ERM proteins, a similar autoinhibitory mechanism was proposed for FAK in which the FERM domain folds over the active site of the kinase domain. The first experiments supporting this mechanism utilized exogenous expression of the FERM and kinase domains separately to show they interact in trans, and that this interaction has a negative effect on functionality [161]. Additionally, expression of individual subdomains F1 and F2 inhibits tyrosine 397 autophosphorylation, indicating that these two subdomains are key for kinase inhibition in FAK [162].

A fragment of FAK containing both the FERM and kinase domains was crystallized, and the structure shows that the FERM and kinase domains do indeed interact (PDB 2JOJ). There are two points of contact between the domains. The most extensive is between the F2 subdomain of the FERM domain and the C-lobe of the kinase. This interaction leads to a 649 Å² area of the kinase domain being buried. This area is highly conserved among various species. This interaction is critical for autoinhibition since mutation of a number of different residues within the buried area creates constitutively active variants of FAK [120]. The second, less intimate, point of contact is between the F1 subdomain and the N-lobe of the kinase through both domains making contact with the linker region. This interaction may also be important in stabilizing the autoinhibited conformation, since mutation of Lys 38, an F1 subdomain residue that appears to interact with the linker, results in a constitutively active mutant [162]. When the FERM domain is bound to the kinase domain part of the linker rests against the F1 subdomain in an anti-parallel β strand interaction. This keeps Y397 approximately 35 Å away from the catalytic cleft of the kinase domain,

preventing intramolecular autophosphorylation. There are several splice variants of FAK that create a longer linker region [163,164]. These variants show elevated phosphorylation and catalytic activity [158]. Further, these variants may exhibit an altered mechanism of autophosphorylation, via an intramolecular rather than *trans* phosphorylation mechanism that may be dependent on the longer linker [158]. Destabilization of the autoinhibited conformation by the altered linker could explain both constitutive activation and mode of autophosphorylation, although this hypothesis has not been tested.

In crystal structures the activation loop of the kinase domain is disordered [120,124]. Based upon the topology of the autoinhibited conformation, Y576 and Y577 are somewhere in the cleft between the active site and the F1 and F2 subdomains of the FERM domain. In the crystal structure of the phosphorylated, active kinase domain (2JoL), the phosphorylated activation loop takes on a β hairpin conformation that is stabilized by hydrogen bonds and electrostatic interactions between the phosphate group on pY577 and the back bone of S580 and A579, and the side chain of R569. This same structure, the phosphorylated activation loop, juts out from the kinase domain, occupying the same space that the FERM domain is present in other crystal structures. This suggests that activation loop phosphorylation can block FERM domain interactions with the catalytic domain. Thus restoration of the autoinhibited state is dependent upon dephosphorylation of the activation loop residues by cellular tyrosine phosphatases. Mutation two lysines to glutamic acids within the activation loop creates a hyperactive mutant, consistent with a role for the activation loop in promoting catalytic activity [165].

Full Length FAK: Putting it all together

While the various structured domains of FAK have been crystallized, the structure of the full length FAK is still unknown. Because of the flexible regions, full length FAK is too flexible to form suitable crystals and the full length protein is too large for NMR studies. Small angle x-ray

spectroscopy (SAXS) studies have yielded some structural insights [166]. SAXS yields lower resolution shapes of molecules or assemblies too large or unwieldy for higher resolution methods. By matching the low resolution shapes from SAXS to the existing FERM domain crystal structures, a FERM dimerization was suggested. Interestingly all existing crystals of the FERM domain contain a similarly shaped FERM dimer [119,120,166]. Additionally, fitting the kinase and FAT domain structures into the SAXS low resolution shape suggested that the FAT domain binds to the F2 subdomain, potentially stabilizing dimers, or holding the autoinhibitory conformation but contacting both the FERM and catalytic domains [166]. Further studies showed that tryptophan 266 is necessary for FERM domain dimerization and FAK association occurs in vitro. Previous studies suggested that transient dimerization was necessary for phosphorylation, which occurs in trans [158]. The SAXS data combined with current knowledge about FAK activation and autophosphorylation suggest that an inactive FAK is curled up upon itself with the FAT domain and proline-rich region wrapped around the FERM-kinase autoinhibited complex. This compact structure would need to unfold in response to activating signals.

F2 the Key to FAK Regulation?

One interesting thing about the SAXS model for full length structure is the dependence on the F2 subdomain for FAT domain binding, more specifically the ²¹⁶KAKTLRK²²² sequence [166]. This sequence has also been shown to be necessary for FAK activation [123]. Additionally, this basic patch is necessary for PIP₂ binding and this interaction triggers FAK conformational change and activation [122]. Further studies into lipid binding suggest that PI(4,5)P₂ interactions lead to FAK FERM domain clustering in vitro [167]. The importance of the ²¹⁶KAKTLRK²²² sequence is further highlighted by its interactions with receptor tyrosine kinases. FAK's interaction with the hepatocyte growth factor receptor Met has been well characterized and depends again on this sequence [38,121]. The GDNF receptor RET also binds to the FERM domain through this sequence,

however this mechanism may differ from PI(4,5)P₂ and Met as unlike the other interactions phosphorylation decreases the affinity of RET for FAK [168]. Overall these studies imply the ²¹⁶KAKTLRK²²² and the rest of the F2 subdomain are vitally important for release of autoinhibition.

Biosensors

Fluorescence resonance energy transfer (FRET) is a phenomenon in which energy is transferred from one excited fluorescent molecule to another which then in turn emits light. This phenomenon has a spatial constraint of 10 angstroms, making it a wonderful tool to investigate molecular interactions. Two separate groups have created FRET based biosensors to measure conformational change in FAK. The conformational biosensor made by the Schaller lab consists of CFP on the N terminus of FAK and citrine at arginine 413 on the N lobe of the kinase. This places the acceptor and donor across the linker from each other. This arrangement, while accurate in indicating conformational change upon FAK activation, is less than ideal as activation, and presumably the open conformation, leads to a reduction in FRET signal [122]. Another group developed a different conformational biosensor which contains YFP at the N terminus of FAK and a CFP inserted at valine 391 in the linker, switching the positions of the donor and acceptor molecules for FRET. The change in fluorophore position is reported to lead to an increase of FRET upon FAK activation in their reported findings, most likely due to a change in fluorophore dipole position as opposed to a decrease in distance [169]. However, reproduction of this biosensor by members of the Schaller lab shows a similar reduction of FRET signal upon activation as the arginine 413 biosensor [Personal communication with W. Fu]. Because of the nature of FRET, studies done thus far with conformational biosensors have not clarified the mechanism of activation. The FERM and kinase domains could be pulled straight apart (domain straightening) or could twist away from each other (twisting switch) upon activation. Since FRET signal will decrease

upon rotation of the fluorophores as well as an increase in distance, these biosensors may not be the tool needed to determine this movement [122,169].

Two other FRET based biosensors have been developed to investigate Y397 phosphorylation. The first phosphorylation biosensor consists of a FAK molecule labeled on the N terminus with CFP and a citrine labeled Src SH2 domain. Since phosphorylation of Y397 is necessary for Src binding, increased FRET signal indicates phosphorylation [122]. This two molecule biosensor allows for observation of FAK activation in a multi-protein context. Another group developed a single molecule phosphorylation biosensor that does not contain full length FAK or Src [170]. This biosensor has a high level of FRET when the Y397 peptide is unphosphorylated. It consists of an ECFP followed by the Src SH2 domain; this is connected by a short linker to a peptide containing the sequence of residues surrounding the FAK Y397 site and YPet. When phosphorylated, it binds to the SH2 domain moving the fluorophores away from each other, decreasing FRET [170]. One advantage of this small phosphorylation biosensor is that it is a reliable measure of endogenous FAK activity as Y397 is the autophosphorylation site. Additionally the addition of other domains can target it to certain areas of the cell. For Example Lyn DRM-targeting motif and K-Ras RGD motif have been added to observe FAK activation near lipid rafts, and the FAT domain has been added to compare FAK activity in the cytosol versus focal adhesions [170,171].

FAK's Place in the Ultra-Structure of Focal Adhesions

Recent experiments utilizing interfering photoactivatable light microscopy (iPALM) clarified the ultrastructure of focal adhesion architecture [172]. This study revealed that focal adhesions are ordered and stratified. Each layer of the stratification is proposed to play a different role in focal adhesion function. FAK and paxillin exist in the layer closest to the membrane, defined as the integrin signaling layer. The other layers are the force transduction layer containing tailin and vinculin, and the actin regulatory layer containing zyxin and VASP. So far iPALM studies have

only been conducted with an N terminally labeled FAK construct [172]. The fluorophore localized to a very narrow range in the focal adhesions suggesting that the bulk of FAK molecules are positioned in the same orientation. N and C terminal labels were utilized on Talin to confirm orientation. It will be interesting to see where the C terminus localizes and if the localization changes in response to activation.

Ligand interactions with the FERM domain of FAK

Growth Factor Receptors

A broad range of extracellular stimuli induce FAK tyrosine phosphorylation and activation, including growth factors. FAK acts as a bridging adaptor protein that links growth factor receptor and integrin signaling pathways to support cell motility. FAK is required in PDGF and EGF induced cell migration, and the N-terminal 402 amino acids of FAK forms a complex containing activated growth factor receptors [36]. Phosphorylation of FAK at Tyr397 is not required for the formation of this complex; however the EGF receptor promotes FAK phosphorylation at Tyr397. Over expression of FRNK disrupts the interaction of FAK with activated EGF receptors. Since FRNK is missing the FERM domain, cell adhesion dependent activation of FAK may be required for interaction with these growth factor receptors. Despite its conservation of sequence, PyK2 does not associate with these activated growth factor receptors [36,173].

A recent study suggests a unique mechanism for interaction between EGFR and FAK utilizing a splice variant of Steroid Receptor Coactivator 3, SRC-3 Δ 4, which binds to steroid receptors and stimulates transcriptional activity [174]. Endogenous SRC-3 Δ 4 co-immunoprecipitates with FAK and the EGF receptor. SiRNA mediated knockdown of SRC-3 Δ 4

partially reduces the co-immunoprecipitation of EGF receptor with FAK, suggesting that SRC-3 $\Delta 4$ may mediate this interaction, although there may be additional molecular mechanisms of interaction between the two kinases. Interestingly, serine and threonine phosphorylation of SRC-3 $\Delta 4$ by PAK is required for binding to both EGFR and FAK. The proposed EGFR/ SRC-3 $\Delta 4$ /FAK complex seems important for cell migration in vitro and metastasis in vivo.

The Met receptor mediates hepatocyte growth factor (HGF) signaling in cells. Stimulation of cells with HGF promotes FAK tyrosine phosphorylation [39]. The phosphorylation of tyrosine 1349 and tyrosine 1356 of Met receptor β -chain is required for its biological activity in promoting cell motility and invasion [162]. A direct interaction between the FERM domain of FAK and the Met receptor, when Met is phosphorylated on tyrosine residues 1349 and 1356, has been reported. This tyrosine phosphorylated peptide is proposed to directly bind several basic residues (²¹⁶KAKTLRK²²²) on the surface of the FERM domain of FAK [121]. As a consequence of this interaction tyrosine phosphorylated Met relieves FERM-domain mediated autoinhibition of FAK resulted in FAK activation. This interaction is important for promoting HGF induced cell invasion [175].

The insulin like growth factor I receptor (IGF-IR) is a transmembrane receptor that initiates a cascade of events that include mitogenic and antiapoptotic responses [176,177]. The relationship between FAK and IGF₁ signaling is complex and likely dependent upon cell context. Different reports demonstrate that IGF₁ induces FAK dephosphorylation through recruiting SHP2 or has no effect upon FAK phosphorylation [178,179]. Others report FAK is important for IGF₁ responses. FAK and IGF-IR were found in the same complex in pancreatic adenocarcinoma cells [35]. This interaction is direct and occurs between the catalytic domain of the IGF-1R and the F2 subdomain of the FAK FERM domain [178]. Additional molecular details of the interaction and characterization of the consequences of binding on the catalytic activity of IGF-1R and FAK have not been fully

elucidated. Cell proliferation stimulated by IGF-1 is dependent on the presence of FAK. Dual inhibition of both FAK and IGF-IR using dominant negative, siRNA and pharmacological approaches decreased cell viability and increased apoptosis in cancer cells [35]

FERM Domain Binding Partners that Regulate the Cytoskeleton

With FAKs role in regulating cell motility, it is unsurprising that cytoskeletal regulators would interact with FAK. Wiskott-Aldrich Syndrome (WAS) protein is encoded by WASL gene. The WAS family of proteins share similar structure and are involved in signaling pathways that regulate the actin cytoskeleton. N-WASP is one family member that is an effector of Cdc42 mediated actin cytoskeleton regulation in cells. FAK interacts with N-WASP and binding occurs between the N-terminal domain of FAK (residues1-400) and N-WASP residues 148-273 [45]. This interaction promotes phosphorylation of N-WASP at tyrosine 256 by FAK, and this phosphorylation can regulate the subcellular location of N-WASP. FAK mediated N-WASP phosphorylation is important in promoting cell migration [45]. Cell migration requires dynamic regulation of actin filaments and focal adhesion complexes in lamellipodia. In these structures, actin filament polymerization is controlled by the Arp2/3 complex, which is regulated by Rac1 and Cdc42 through WASP proteins. The FERM domain of FAK is also believed to interact with the Arp2/3 complex, and thus may scaffold N-WASP and the Arp2/3 complex to control actin polymerization in lamellipodia [46]. Interestingly, phosphorylation of tyrosine 397 of FAK impairs the interaction of FAK with Arp 2 and Arp3, suggesting that FAK autophosphorylation triggers the release of the Arp2/3 complex, which promotes initiation of nascent lamellipodia formation and cell spreading [46,180].

FAK also interacts with C terminal region of myosin in cardiomyocytes. First identified by a yeast two hybrid screen using the N terminal portion of FAK as bait, this interaction inhibits FAK phosphorylation and activation [70]. Interestingly, FAK is localized to the sarcomeric A band in

cardiomyocytes and pull down studies lead to the estimation that 40% of FAK is bound to myosin in unstimulated cardiomyocytes [70]. In vivo studies showed that cardiac pressure overload reduced this interaction and increased FAK phosphorylation. A later study used in silico modeling and small angle x-ray scattering (SAXS) to further identify the characteristics of this interaction [79]. The coiled coil of myosin rests in a cleft formed by the three subdomains of FERM opposite the side that the linker binds to. Mutational studies further identified the sequence ¹⁵⁸EIADQVDQE¹⁶⁶ on the FERM domain as the sequence important for this interaction [79]. Additionally, cyclic stretch of cardiomyocytes reduces this interaction and a FERM mimetic peptide can act as a decoy for myosin, leading to the activation of FAK [79].

Other FERM Binding Partners

It was reported that the N-terminal domain of Ezrin interacts with amino acids 1-376 in the FERM domain of FAK. This interaction may promote activity of FAK through increasing Tyr397 phosphorylation. Ezrin-dependent activation of FAK is not linked to cell-extracellular matrix adhesion [181]. The molecular details of this interaction and significance of this mechanism of regulation are currently unknown.

FAK also interacts with the PH domain of Etk directly through the FERM domain to promote endothelial cell or epithelial cell migration. Activated FAK may play a critical role in increasing the activity of Etk since the FAK Y397F mutant lacks the ability to activate Etk. The FAK sequence mediating the interaction is unknown, but is presumably unique to FAK since Pyk2 fails to bind Etk. The Y40 and E42 residues in the PH domain of Etk are critical for Etk activation by FAK [182] Y40 is buried in the lipid binding pocket and E42 is on the surface of the PH domain in the proximity of the lipid binding site (based upon the structure of the highly related Btk PH domain). Under some scenarios, signaling via other tyrosine kinases might promote the association of FAK and Etk. He et al. for example, demonstrate that the tyrosine kinase inhibitor, AG879 which

is an inhibitor specific for ErbB2 and VEGF receptor FLK-1, inhibits the association of FAK and EtK through activation of PAK1 [183]. While this is an intriguing mechanism of regulation, there are several outstanding questions including the relationship between lipid binding and FERM domain binding, and if lipid- and FERM-mediated activation occurs via similar mechanisms.

RIP is a family of proteins named receptor interacting proteins. They are serine/threonine kinases that contain an N-terminal kinase domain and a C-terminal death domain. The death domain of RIP1 interacts with a number of receptors that function in the induction of apoptosis. RIP1 acts downstream of TNF-R1 to control NF- κ B activation and thus plays a role in determining whether cells survive or undergo apoptosis [184]. RIP interacts with the N-terminal domain of FAK both in vivo and in vitro. RIP provides proapoptotic signals regardless of whether cells are suspended or attached because RIP^{-/-} cells were resistant to the effects FAK dephosphorylation and degradation. Binding to FAK directly shifts the FAK-RIP balance towards resistance to apoptosis and promotes survival in FAK overexpressed cancer cells. This is postulated as one mechanism used by FAK to generate a strong survival signal in tumor cells [185].

Kadare et al found that the protein inhibitor of activated STAT1 (PIAS1) interacts with the N-terminal domain of FAK in a yeast two-hybrid screen [186]. They identified residues 403-651 in the PIAS1 C-terminal domain as the FAK binding site. This interaction would potentially induce sumoylation of FAK at Lysine 152 and increase the phosphorylation level of FAK at tyrosine 397 in cells. However, lysine 152 is buried in the structure of the FAK FERM domain, in the groove between the F1 and F2 subdomains. In the autoinhibited FAK conformation, Lysine 152 lies between the FERM and Kinase domains and is further inaccessible for sumoylation in this conformation. Considerable structural rearrangement would be required to facilitate FAK sumoylation at this site. But if sumoylation could occur, the SUMO moiety would certainly interfere with assembly of the

autoinhibited conformation, consistent with the observed elevation in phosphorylation in the sumoylated population of FAK.

Interactions with the Kinase Domain

As the kinase domain serves a catalytic function and most of the structural features are required to carry out that function, it is unsurprising that there are few interacting proteins with this domain. One protein does however interact with the catalytic domain in a very unique way. FIP200 is the FAK family kinase-interacting protein of 200 kD [187]. It was originally identified in a yeast two hybrid screen for Pyk2 interacting proteins. The C-terminal domain of FIP200 binds to the catalytic domain of Pyk2 and impairs the activity of Pyk2 3-5 fold in vitro [187]. In transient expression assays, FIP200 inhibited Pyk2 phosphorylation and the induction of apoptosis, suggesting FIP200 can regulate biological responses to Pyk2. FIP200 also binds FAK. The C-terminal domain of FIP200 binds the N-terminal domain of FAK, while the middle and N-terminal domains of FIP200 both bind the catalytic domain of FAK [188]. This interaction impairs kinase activity in vitro. FIP200 complexes with FAK in cells in suspension and appears to dissociate upon integrin dependent cell adhesion, particularly to fibronectin. Interestingly, FIP200 appears to inhibit FAK-dependent phosphorylation of paxillin and Shc, but does not affect FAK-dependent phosphorylation of p130cas and Grb7, in transiently transfected cells [188]. For a number of reasons, FIP200 is an intriguing binding partner of FAK and Pyk2. The potential to tether the FERM and kinase domains together is an exciting one. However, this interaction may be more specific to Pyk2, as conditional knockout of FIP200 suggests that in vivo it is important for Pyk2 regulation but not FAK regulation [189].

Proteins Interacting with Focal Adhesion Targeting Domain

The C-terminal FAT domain was originally defined as the sequence responsible for correct subcellular localization of FAK to focal adhesions [139]. Its simple 4 alpha-helical bundle structure

serves as a binding site for a number of proteins and is now established to engage multiple transmembrane receptors and downstream substrates for phosphorylation.

The first FAT domain binding partner identified was the focal adhesion associated protein, paxillin [190–192]. FAK binds to two peptide motifs (LD motifs) on paxillin and in turn has two binding sites for paxillin in the FAT domain. Paxillin binding is one mechanism by which FAK is localized to focal adhesions. Paxillin is also a substrate for FAK, and phosphorylation of paxillin on Tyr¹¹⁸ recruits additional signaling molecules into complex to activate downstream signaling [193]. Paxillin also scaffolds signaling molecules in a phosphotyrosine-independent manner, including PKL/Git2 (paxillin kinase linker) and β -pix (a guanine nucleotide exchange factor for Cdc42 and scaffold for Rac and PAK). The FAK/paxillin complex also facilitates tyrosine phosphorylation of PKL/Git2 and β -pix [194,195]. Tyrosine phosphorylation of β -pix impacts Rac recruitment and tyrosine phosphorylation of PKL regulates cell polarization, lamellipodia stability and directional persistence of migrating cells [195,195].

Growth factor receptor bound protein 2 (Grb2) contains an SH2 domain and a pair of SH3 domains [196]. The binding of Grb2 to FAK is dependent on phosphorylation of FAK at tyrosine 925, which creates a Grb2 SH2 domain binding site. This phosphorylation/binding site resides in the first alpha helix of the FAT domain and conformational change of the FAT domain must precede phosphorylation and Grb2 binding. The FAK/Grb2 interaction is induced by integrin receptors and is coupled to activation of the Ras signaling pathway and downstream MAPK [196].

There is another group of proteins related to transcription signaling pathway which is regulated by FAK activity. Over expression FAK causes activation of Stat1 which is one of the signal transducer and activator of transcription (STAT) pathway proteins. STAT1 interacts with C-terminal of FAK directly [197]. The consequences of STAT1 binding are apparently a reduction in

cell adhesion with an increase in cell motility through competition for focal adhesion localization of FAK.

The endocytosis motif in the cytoplasmic domain of CD4 is similar to the LD motifs of paxillin. The signature aspartic acid is not found in CD4, but the endocytosis motif is predicted to form an amphipathic alpha helix similar to the LD motifs. A peptide mimicking the CD4 motif (amino acids 405-417) binds the FAT domain of FAK with similar thermodynamic properties as the paxillin LD4 motif. Both interactions show two binding sites on the FAT domain, are entropy driven, and exhibit similar affinities [198]. Mutation of this sequence in the CD4 endocytosis motif abolishes binding. The structure of this CD4 peptide in complex with the FAT domain of FAK has been solved (PDB 3B71). The amphipathic alpha helix docks with two hydrophobic patches of the FAT domain generally reminiscent of the interaction of paxillin LD motifs [198]. Interestingly, CD4 residues contacting the FAT domain are also critical for CD4 interactions with Lck, which binds with significantly higher affinity. It is proposed that disruption of the CD4/Lck complex, e.g. by cellular stimulation with the HIV gp120 protein, is required for the assembly of a CD4/FAK complex [198].

Convergent lines of investigation led to the discovery that FAK plays a role in neurons to regulate axonal outgrowth and guidance, and in oligodendrocytes to control branching. FAK directly associates with the cytoplasmic domain of neogenin and DCC (deleted in colon cancer) [199–201], which serve as receptors for netrin 1, an extracellular ligand controlling axonal pathfinding and stimulating oligodendrocyte branching. The FAT domain of FAK mediates this interaction with an LD-like motif in the C-terminal domain of neogenin/DCC [199–201]. Intriguingly, these receptors bind FAK but not Pyk2, but the basis of selective binding is unknown [199]. This receptor/FAK interaction is essential for netrin 1 induced FAK tyrosine phosphorylation and FAK is required for netrin 1 induced axonal outgrowth and guidance [199–201]. More recent

investigation into netrin 1 growth cone guidance implicated the DCC/FAK interaction in a mechanosensory role [202]. In this model, netrin activates DCC, which is bound to the FAK FAT domain. The FERM domain is connected to the actin cytoskeleton through an interaction with an adaptor, possibly Ezrin, allowing traction force to activate FAK through separation of the FERM and kinase domains [202]. In addition to controlling neuronal outgrowth, netrin 1 also promotes specific translation of the kappa opioid receptor in dorsal root ganglion neurons. FAK and the associated adaptor protein, Grb7, have been identified as components of this signaling pathway [203,204].

P190RhoGEF was identified as a FAK FAT domain binding partner in a yeast 2-hybrid screen, and endogenous p190RhoGEF co-immunoprecipitates with endogenous FAK [44]. The p190RhoGEF sequence extending from residue 1292 to 1301 is required for FAK binding, but the extent of the binding site has not been defined [44]. This sequence is not similar to the LD motifs of paxillin and thus binding apparently occurs via a unique mechanism, which remains to be determined. Cell adhesion to laminin or fibronectin induces tyrosine phosphorylation of p190RhoGEF, which correlates with activation of Rho, and both of these signaling events are dependent upon FAK [44,205]. This signaling pathway is proposed to regulate axonal branching in Purkinje and hippocampal neurons by impairing branching and promoting axonal retraction via activation of Rho [15].

Development of FAK Inhibitors

Based upon the expression of FAK in human tumors, the correlation of FAK expression or amplification with disease outcome and the demonstration that FAK functions to promote tumorigenesis and metastasis in animal models, there is considerable interest in FAK's potential as a therapeutic target. Several techniques have been used for the inhibition of FAK. Antisense oligonucleotide and shRNA interference approaches have been used to inhibit FAK. However

limitations in effective drug delivery systems for these reagents exclude them from use therapeutically [206,207]. Several groups have reported the development of small molecule inhibitors of FAK catalytic activity and others have suggested additional therapeutic strategies. Currently the most promising FAK inhibition strategies are small molecule inhibitors .

The Genomics Institute of the Novartis research Foundation and the Novartis Institute for BioMedical Research used rational drug design to produce Compound 1 [208]. This pyrrolo[2,3-d]pyrimidine uses a five membered ring to bridge the four and five phosphates of the pyrimidine. The results of molecular modeling and co-crystal structure analysis suggested that improvements to specificity and inhibition could be made by adjusting the side groups [209]. The modifications led to compound 32 which contains a 2-pyrimidine ring and extended carboxy group. This carboxy group forms a salt bridge with Lys 454 and increases specificity to an IC₅₀ of 4nM.

Another Novartis drug that is further along in characterization is TAE226. TAE is a bis-anilino pyrimidine that interacts with the kinase hinge region [210]. It has an IC₅₀ between 100 and 200 nM [211]. Two hydrogen bonds are formed between Cys 502 and the pyrimidine and 2-methoxyaniline of TAE 226. Additional hydrophobic contacts between the pyrimidine and Ala 452 and Leu 553 as well as interactions between the 2-methoxyaniline and Ile 428 and Gly 505 hold the inhibitor securely in place. The binding of the inhibitor in this way stabilizes the DFG motif in an unusual helical conformation. This flexibility is due to a glycine directly before the DFG motif (PDB 2JKK). While this glycine is highly conserved in FAK it is rare in most other kinases [210]. However this glycine is present in the IGF-IR which allows TAE226 to inhibit it as well. TAE226 increases survival rates in rats with glioma and ovarian cancer xenografts, however because it also inhibits IGF-IR it is unsuitable for trial in human subjects [211,212].

Another FAK kinase inhibitor is PND-1186 [213]. It has an IC of 1.5 nM for FAK and has the unique characteristic of not having an inhibitory effect on FAK phosphorylation in adherent cells

[213]. PND 1186 increases apoptosis in breast and ovarian carcinomas as well as reduces tumor associated inflammation [213,214]. A twice daily oral dose of 150mg/kg prevented tumor growth and metastasis to the lung in breast cancer xenografted mice [214].

Pfizer developed two separate FAK inhibitors, PF 573,228 and PF 562,271. Both compounds were discovered through a combination of high throughput screening, structure based design, and conventional medicinal chemistry and both are methane sulfonamide diaminopyrimidines [215,216]. PF 573,228 has an IC₅₀ of 4nM for FAK and 1μM for Pyk2 and is very specific to these two kinases [216]. PF 573,228 inhibited cell spreading and migration as well as reduce cell growth. It did not significantly reduce Tyr 397 phosphorylation. PF 562,271 has been characterized to a greater extent than its counterpart and its crystal structure has been solved (PDB ID 3BZ3) [215]. It forms three hydrogen bonds, two with Cys 502 and one with Arg 426. The sulfonamide group interacts with the DFG motif, with the oxygen hydrogen binding to Asp 564. This moves Asp 564 upwards and away from the ATP binding site, and creates the same helical DFG motif conformation seen upon binding of TAE 226 [210,215]. PF 562,271 inhibits FAK phosphorylation in tumors as well as increases apoptosis of tumor cells. Nude rats injected with MDA-MB-231 cells, mimicking a secondary, post-metastatic tumor site, were given an oral dose of 5mg/kg [102]. This dosage prevented the growth and spread of the bone metastases and reversed tumor induced bone loss through inhibition of Pyk2. Similar results were seen with a combined dosage of PF 562,271 and the antiangiogenic drug, sunitinib in a rat xenograft model with hepatocellular carcinoma cells [101]. Recently, Verastem acquired PF-04554878 and renamed it VS-6063 [217]. This company has worked with the FAK inhibitor throughout clinical trials, including Phase I studies as a combination with Paclitaxel ovarian cancer, and Phase II studies in non-small cell lung cancer and mesothelioma.

The newest FAK inhibitor is a small molecule inhibitor that also binds to the ATP binding pocket in FAK developed by GlaxoSmithKline, called GSK2256098 [218]. It is very selective, with

1000 fold greater selectivity for FAK over Pyk2 [219]. It inhibits both growth and survival in pancreatic ductal adenocarcinoma cells, however the extent of the effect is variable across the 6 different PDAC cell lines tested [220]. Despite these variable results, GSK2256098 has passed Phase I clinical trials in healthy individuals and patients with solid tumors [221]. Additionally, the results of Phase I dose escalation study showed a promising effect in patients with solid tumors [219]. Three more Phase I combination therapy studies with the drug and other cancer therapies are underway, as well as a Phase I study in hypertensive patients [221]

A truly unique approach was taken in the development of Compound 14 [222]. A search of the National Cancer Institute's compound data base was combined with molecular modeling to identify candidates. Compound 14, 1,2,4,5-benzenetetraamine tetrachloride, is very small and binds directly Tyr 397. In vitro studies showed that it decreased phosphorylation of Tyr 397, cell adhesion, cell viability and blocked tumor growth. However no specificity studies were completed, and compound 14's structure suggests many off target effects.

Since the FAT domain alone is also a potent dominant negative mutant [111], this domain is considered a potentially significant therapeutic target to impair FAK signaling. A phage display strategy was utilized to identify peptides that bind to the C-terminal domain of FAK. This resulted in discovery of the AV₃ peptide. The AV₃ peptide can displace FAK from focal adhesions and impair FAK function [223]. These studies were extended using an in silico screen for compounds that bind to the FAT domain at the AV₃ binding site. The compound, chlorpyramin hydrochloride [N-(4-chlorobenzyl)-N',N'-dimethyl-N-pyridin-2-ylethane-1,2-diamine], impaired FAK signaling in cells, inhibited proliferation and induced apoptosis [224]. The crystal structure of this compound bound to the FAT domain has been solved and deposited in the protein data bank (PDB ID 2RA7). The compound docks to the alpha helix 1/4 side of the FAT domain, making extensive hydrophobic contacts and H-bonding to Ser 939 (in helix 1) and His 1025 (in helix 4). The drug binding site

partially overlaps the paxillin LD motif binding site, and His 1025 is one of the basic residues flanking the hydrophobic patch on this side of the FAT domain. Thus this compound potentially perturbs paxillin binding and might effectively displace FAK from focal adhesions, since the paxillin binding site on the helix 1/4 surface of the FAT domain appears most important for localization [149]. These hypotheses have yet to be addressed.

Phosphatidylinositol-(4,5)-bisphosphate Signaling in Focal Adhesions

The importance of PI(4,5)P₂ in focal adhesion signaling and cytoskeleton regulation has been well established. As a precursor for the second messengers PI(3,4,5)P₃ inositol (3,4,5)-trisphosphate, and diacylglycerol, its levels help to regulate cytoskeleton-membrane interaction [225]. PIP5K1γ, a kinase capable of creating the PI(4,5)P₂ is recruited by the FERM domain of talin to focal adhesions [226]. Once localized to adhesions, PIP5K1γ creates a localized increase of PI(4,5)P₂ [227]. This FA specific pool of PI(4,5)P₂ is necessary for focal adhesion formation as well as force generation. Localized PI(4,5)P₂ is necessary for the proper association of talin and integrins [228]. PI(4,5)P₂ also plays a role in the dimerization of vinculin and the vinculin sequestration necessary for proper adhesion function [229,230]. PIP5K1γ is an upstream regulator of FAK activity, which is unsurprising given the role of PI(4,5)P₂ binding in inducing conformational change [122,167]. Another lipid kinase PIPKIβ, could also increase the local levels of PI(4,5)P₂, leading to its role in regulating focal adhesion disassembly through FAK activation and integrin endocytosis [231]. Overall these studies show that the localized ebb and flow of PI(4,5)P₂ regulates proteins necessary for the formation and disassembly of focal adhesions.

Conclusion

The objective of this thesis was to elucidate more information regarding the mechanism of activation for FAK. Previous research has highlighted the importance of the FERM domain, particularly the F2 subdomain in regulation of FAK activation. Additionally, PI(4,5)P₂ and phosphotyrosine binding are implicated in triggering a more active FAK molecule through the release of the autoinhibited conformation. The following studies build on the previous literature to better classify FAK activation in the context of disease, as well as the inter- and intramolecular interactions that regulate the autoinhibited and open, active conformations.

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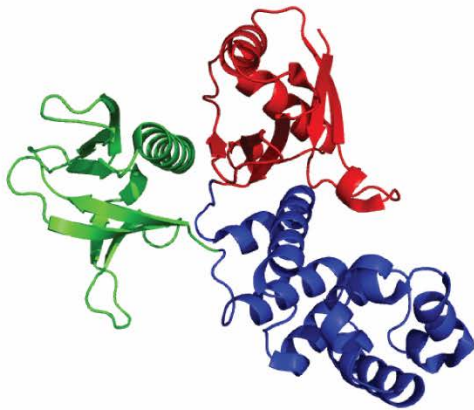
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Figure 1: The structure of FAK. A) A cartoon representation of the domains of FAK organized linearly. Tyrosine and serine phosphorylation sites, as well as PxxP binding motifs are highlighted. The location of the KAKTLRK sequence is highlighted by the dark blue box. A) The crystal structure of the FERM domain. F₁ is shown in red, F₂ in blue and F₃ in green. B) The crystal structure of the kinase domain. The ATP binding site holds a stick representation of an ATP analog. C) The crystal structure of the FAT domain. The cooler colors start at the N terminus and the warmer colors end at the C terminus. Tyrosine 925 is highlighted in yellow. (Adapted from Hall JE, Fu W, Schaller MD (2011) Focal adhesion kinase: exploring Fak structure to gain insight into function. *Int Rev Cell Mol Biol* 288: 185-225 with editor permission.)

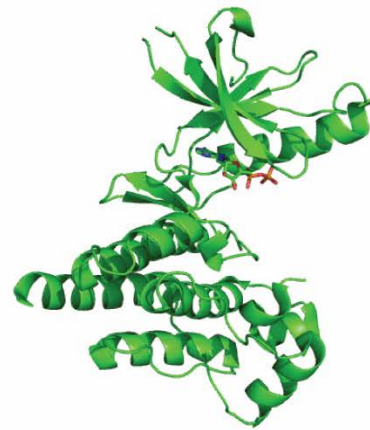
A.



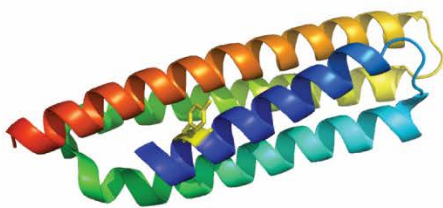
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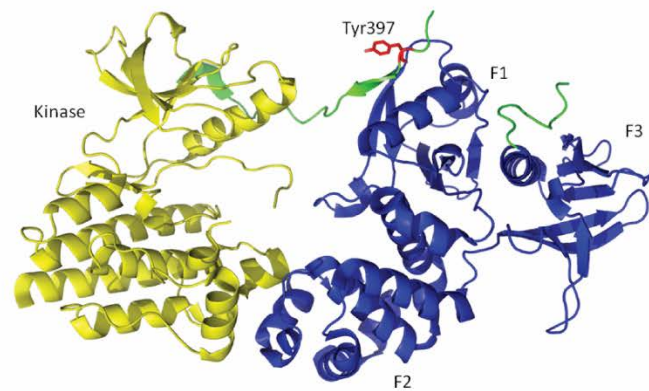


Figure 1

Chapter 2: Investigation of mutations found in patient samples and basic residues on the FERM domain

Abstract

Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase found in focal adhesions. It assists in regulation of the assembly and disassembly of these adhesions during cell migration and plays a role in integrin signaling and cell survival. FAK also integrates signals from several growth factor receptors and has a scaffolding function that aids in integration of signaling pathways. It is overexpressed in many cancers including colon cancer, ovarian cancer, and glioma. Additionally, the FAK gene is amplified in breast cancer. Higher levels of FAK or increased catalytic activity can be linked to poor prognosis and FAK is implicated in metastatic progression. FAK catalytic activity is regulated through an autoinhibitory mechanism, in which the N-terminal FERM domain folds over the active site of the kinase domain and is held in place by hydrophobic and charge interactions. Previous studies have indicated a basic patch on the FERM domain in binding to small ligands, such as $\text{PI}(4,5)\text{P}_2$, and signaling for FAK activation through growth factor receptors such as Met receptor. Binding of these ligands to the FERM domain disrupts this interaction and releases autoinhibition. The mechanism leading to this release of autoinhibition is not fully understood. To this aim, two sets of mutations were assessed for their effect on release of autoinhibition. The first set of mutants were identified in patient samples or tumor cell lines. These mutations could give insight into a novel regulatory mechanism present in disease states. The second were designed based on FAK structural data to provide insight into the physiological method of activation. While it was hypothesized that mutating these residues would lead to a more active FAK, results suggest that FAK activity is not increased by mutation in cancer but by overexpression or upstream signals. Additionally, it was found that FERM domain residues may contribute to the maintenance of the autoinhibited and open conformations.

Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase with an additional scaffolding function. It plays an integral part in assembling signaling complexes in focal adhesions, which are the sites of contact between the cell cytoskeleton and underlying extracellular matrix. While association with the matrix through integrins is a major mechanism of FAK regulation, FAK also interacts with many growth factor receptors and participates in signaling downstream in these pathways [1,2]. Because of its participation in focal adhesion signaling and growth factor signaling, FAK serves three major roles in the cell: regulation of motility, promotion of cell survival, and control of proliferation.

Given that aberrant growth, survival and motility are hallmarks of metastatic disease, it is not surprising that FAK plays a significant role in the pathological state of cancer [3]. FAK expression is increased in many cancers including melanoma, glioma, prostate, liver and ovarian cancers [4,5]. Additionally, its gene is amplified in breast cancer [6]. Increased FAK expression or activity has been linked to a higher rate of metastasis as well as poor prognosis in a number of cancer types, including breast and ovarian cancers [4,7]. Determining the mechanisms of regulation of FAK is important for understanding the progression of these diseases and the development of new therapies [8].

FAK is a multidomain protein with three distinct structured domains. At the N terminal end, FAK contains a band 4.1/Ezrin/Radixin/Moesin (FERM) domain [9]. Like most FERM domains it is a three-lobed domain comprised of the subdomains F1, F2 and F3, however the FAK FERM domain is structurally distinct from its family members. The FAK FERM domain has a different arrangement of the subdomains and several areas where the exposed residues vary greatly from other FERM domains giving FAK the potential for unique ligand interactions [10]. The FERM domain is connected to the kinase domain by a short unstructured linker, which contains tyrosine

397, the FAK autophosphorylation site [11]. C-terminal of the kinase domain there is a large unstructured region that contains several proline rich sequences, SH₃ domain binding sites, and serine and tyrosine phosphorylation sites [12]. This region plays a large role in FAKs scaffolding activity, with several phosphorylation sites and PxxP motifs. The C terminus is the Focal Adhesion Targeting (FAT) domain. This antiparallel four helix bundle binds to paxillin through both hydrophobic and charge-charge interactions and anchors FAK in the focal adhesions [13]. Recently several crystal structures of domains of FAK have been solved, providing further insight into the structural nuances of these three domains [10,13,14].

FAK activity is partially governed through autoinhibition. In the autoinhibitory conformation, the FERM domain folds over the kinase domain blocking the active site [14]. The largest point of contact between the FERM and kinase domains is between the F2 subdomain of the FERM domain and the large C-terminal lobe of the kinase domain. Here hydrophobic interactions as well as charge-charge interactions hold the two domains together. Additionally the linker between the two domains forms an anti-parallel β sheet with residues in the F1 subdomain. This pulls the F1 subdomain close to the N-terminal lobe of the kinase domain [14].

Overall the mechanism for release of autoinhibition is not well understood. There is some evidence that release of autoinhibition can be triggered by binding of an acidic ligand to a basic patch on the surface of the F2 subdomain [15,16]. Phosphorylation of tyrosine 397 in the linker, and possibly recruitment of Src into complex, could also break the β sheet interaction at the F1 subdomain. We set out to determine the mechanism of release of autoinhibition in order to better understand the increase of FAK activity in cancer. The following studies used two different strategies for identifying potential residues that could play a role in FAK activation. The first approach was by identifying residues mutated in human tumor samples from the Catalogue of Somatic Mutations in Cancer (COSMIC) database. The COSMIC database is maintained by the

Sanger Institute and is a compilation of mutations found in patient samples (<http://cancer.sanger.ac.uk/cosmic>) [17]. The second was a rational design approach that identified residues in the FERM domain that interacted with the kinase domain. The residues R184, K190 and K191 are in the alpha helix neighboring the ²¹⁶KAKTLRK²²¹ sequence. The side chains of these residues formed a salt bridge with residues on the kinase domain. Interestingly these residues and the corresponding helix had different peptide backbone and side chain positions in the crystal structures of FERM alone (PDB:2AL6) vs the FERM-Kinase complex (PDB:2JoJ) [10,14]. All of these residues were basic with the potential to bind to an acidic ligand such as phosphotyrosine or phospholipids. To neutralize the charge on these residues they were mutated to alanine.

Materials and Methods

Molecular Biology

FAK mutants were created using a modified version of the Quickchange (Agilent) mutagenesis protocol using CYFAK413 plasmid as a template. Primers for site-directed mutagenesis were designed to substitute residues at the sites of interest. Sequence analysis verified the presence of the intended mutations and that no unintended mutations were present. (ACGT inc).

Cell culture and reagents

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and MDCK (ATCC, Manassas, VA) cells were maintained in Minimum Essential medium with 10% fetal bovine serum. Cells were transfected with Lipofectamine 2000 or LTX (Life Technologies Corp., Grand Island, NY) using manufacturer's recommendations. Small molecule inhibitor SU11274 was purchased from Tocris Biosciences (Minneapolis, MN). Recombinant human Hepatocyte Growth Factor (Sigma, St. Louis, MO) was used at 20 ng/ml. Antibodies used were anti-FAK 4.47 monoclonal antibody (Millipore), anti-phospho pY397 FAK (Life Technologies), anti-phosphotyrosine 4G10 (Upstate), anti-cMet C-28 (Santa Cruz), anti-GAPDH (Millipore), anti-E-cadherin (BD Biosciences)

Protein Analysis

Cells were lysed in ice-cold modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.3], 150 mM NaCl, 1% IGEPAL, 1% Nonidet P-40, 0.5% deoxycholate, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM vanadate). Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid assay (Pierce). Lysates were boiled in Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) and 15 μ g of total protein was analyzed by Western blotting. A FAK antibody (4.47), a phosphotyrosine antibody (4G10) and FAK phosphospecific antibodies (PY577, PY397 and

PY861) were used as primary antibodies for Western blotting. Horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies (Millipore) were used to detect primary antibodies using Immobilon Western HRP chemiluminescence substrate (Millipore). Quantification of blots was performed with ImageJ (<http://rsb.info.nih.gov/nih-image>). Developed films were scanned as TIFF images in 8-bit grayscale format at 600 dpi. The lanes were defined using the rectangular select tool and the Analyze → Gels → Select First Lane/Select Next Lane function. Densitometry measurements were calculated using Analyze → Gels → Plot lanes. Band peak was defined from background using the straight line tool then the area of the band peak was calculated using the Wand tool. (Protocol can be found at <http://rsb.info.nih.gov/nih-image/manual/tech.html#analyze>.) Band intensities of the phosphotyrosine blots were divided by the band intensities of the FAK blots and all wild type samples were normalized to one.

Kinase assay

HEK293 cells were transfected with FAK biosensors were lysed after 24 hours after transfection with modified modified radioimmunoprecipitation assay (RIPA) buffer. Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid assay (Pierce). Lysates were diluted to yield a total protein concentration of 100µg/µl. 2µl of monoclonal antibody BC4 for FAK was added to the lysates and incubated for 1 hour at 4°C. Protein A/G agarose beads were added to the lysate and were incubated for 1 hour at 4°C with rocking. Beads were washed twice with lysis buffer and then twice with kinase buffer (50mM tris-HCL pH7.4, 5mM MnCl₂, 5mM MgCl₂). Beads were resuspended in 25µl kinase buffer containing 2.5µg of a GST fusion protein containing the FAK y397 peptide sequence and 25µM ATP. The reaction was incubated for 5 minutes at room temperature then stopped by adding 7.5µl of Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue). The sample was Western Blotted utilizing pY20 (Millipore) and 4.47.

Fluorometric measurements of FRET

For FRET measurements in the spectrofluorometer, HEK 293 cells expressing FAK constructs were washed with phosphate buffered saline (PBS) followed by detachment using PBS containing 0.02% EDTA and transferred to a cuvette. The cell suspension was excited at 425nm and emission scans (450nm-550nm) were acquired. The spectra of different samples in each experiment were normalized to the CFP emission of the wild type biosensor spectrum.

For FRET measurements in the BioTek Synergy H4 Hybrid plate reader untransfected 293 cells and cells expressing FAK and/or cMet constructs were plated in a black clear bottomed 96-well plate (BD Falcon). Full well emission scans were done with excitation and emission read pairs of 425nm/470nm (CFP excitation/CFP emission), 470nm/530nm (YFP excitation/YFP emission), and 425nm/530nm (CFP excitation/YFP emission; FRET signal). The cells were excited at 425nm and emission scans (450nm-550nm) were acquired. The background fluorescence of untransfected cells was subtracted and spectra of different samples in each experiment were normalized to the CFP emission of a reference spectrum from a wild type biosensor. FRET ratios were calculated for each well using both the well scan and emission scan data as an internal control.

Lipid Binding

Lipid binding was assessed by co-sedimentation with large, unilamellar vesicles (LUV). The ability of the catalytic domain to bind PI(4,5)P₂ was analyzed using lipid vesicles containing 50%PC/40%PE/10% PI(4,5)P₂ (experimental) or 60%PC/40%PE (control). Vesicles were prepared by mixing chloroform dissolved phospholipids (Echelon) in appropriate ratios. The mixture was dried using a speed vacuum for 15 minutes. The dried lipid cake was suspended into a lipid binding buffer (20 mM HEPES [pH 7.5], 2 mM dithiothreitol, 250 mM NaCl) to a final concentration of 2.5 µg/ul [18]. The lipid suspension containing large multilamellar vesicles (LMV) was passed eleven times through a 100 nm filter using an Avanti mini-extruder set to ensure a homogenous suspension

of LUVs. Four micrograms of glutathione S-transferase (GST) fusion protein was incubated with 250 µg of lipid vesicles on ice for 1h. The mixtures were centrifuged at 100,000 x g for 1h at 4 C°. The supernatants were collected, mixed with Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% S.D.S, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled. The pellets were resuspended in Laemmli sample buffer and boiled. The samples were analyzed by S.D.S-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. Quantification of gels was performed with ImageJ (<http://rsb.info.nih.gov/nih-image>). Images of gels were converted to TIFF images in 8-bit grayscale format at 600 dpi. The lanes were defined using the rectangular select tool and the Analyze → Gels → Select First Lane/Select Next Lane function. Densitometry measurements were calculated using Analyze → Gels → Plot lanes. Band peak was defined from background using the straight line tool, then the area of the band peak was calculated using the Wand tool. (Protocol can be found at <http://rsb.info.nih.gov/nih-image/manual/tech.html#analyze>.) Band intensities of the supernatant and pellet lanes were divided by the sum of the band intensities of the supernatant and pellet.

FRET measurements in response to HGF stimulation

MDCK cells transfected with FAK biosensor constructs were plated onto collagen coated (50 µg/ml) glass bottom microwell dishes (MatTek Corp, Ashland, MA). Dishes were placed in a CO₂ and temperature controlled environmental chamber mounted onto a Nikon Swept Field confocal. Cells expressing biosensors were visualized with a Nikon 40x oil objective and imaged using Nikon Elements FRET module with exposure times of 200ms for YFP, and 800ms for CFP and FRET channels (Hahn ref). Background subtraction was performed followed by generation of a ratio image of CFP divided by FRET channels. Cells were outlined and intensity averages of each channel along with FRET ratios were recorded using the Nikon Elements Analysis software.

Results

COSMIC Mutations Have No Effect on FAK Activation

Several FAK mutations are present in the COSMIC database. Although the overall mutation rate of FAK in cancer is extremely low (1%), several of the mutations occurred at intriguing sites within the FAK structure. Further analysis of these mutants could provide insight into FAK activation. The first mutation, F147C, was identified in a non-small cell lung cancer patient. It is located in the α_2 helix of the F2 FERM subdomain and makes contacts with side chains of residues in the α_1 and α_2' helices. The F2 subdomain is the largest point of contact with the kinase domain in the autoinhibitory conformation, making a structural disruption of this subdomain likely to cause a disruption in autoinhibition. The second mutation, D402V, was also found in a non-small cell lung cancer patient. This residue is in the linker very near the Y397 and Y407 phosphorylation sites. The mutation also removes a charged side group from the linker. This could disrupt FAK autophosphorylation at Y397 and Y407. The third mutation, A590V, was identified in human glioma patient. It is located in the kinase domain shortly past where the activation loop rejoins the C lobe. A disruption here could cause a change in activation loop conformation, inhibiting autoinhibition (Figure 1). Three different FAK mutants were created based on these mutations.

Previously, a FRET based biosensor of FAK was developed, CYFAK₄₁₃ [19]. This construct consists of a CFP at the N terminus of FAK and YFP inserted in the linker near the kinase domain. When FAK is in the autoinhibited conformation, the acceptor and donor fluorophores are in close proximity and the FRET signal is high. Release of the autoinhibitory conformation causes a reduction in FRET. Using a modified Agilent Quickchange protocol the CYFAK₄₁₃ biosensor was mutated to create three constructs containing residue substitutions mimicking the mutations in

the COSMIC database. These mutations were analyzed to determine if they resulted in altered FAK conformation and activation.

To determine levels of FAK protein produced and phosphorylation, populations of HEK293s expressing each construct were lysed and lysates were analyzed by Western blotting (Figure 2 A & B). A mutant of FAK, Y180A/M183A, that is incapable of maintaining the autoinhibited conformation was used as a control in all experiments. FAK phosphorylation at Tyr397 was not affected by the F147C and D407V mutants. FAK expression levels and Tyr397 phosphorylation were significantly decreased in the A590V mutant. To confirm that the changes in phosphorylation were due to FAK autophosphorylation activity and not the disruption of an upstream activating signal, *in vitro* kinase assays were performed. FAK was immunoprecipitated from transiently transfected HEK293 cells and incubated with a fusion protein substrate which mimics the Y397 phosphorylation site for 5 minutes. The reaction was then blotted for FAK and phosphotyrosine. The catalytic activity of the F147C and D407V mutants were similar to that of the wild type protein (Figure 2 C & D). There was no detectable catalytic activity in the A590V mutant. Thus none of the mutants lead to a more active kinase, with two having activity comparable to wild type and one being catalytically dead.

Despite the lack of change in kinase activity in an *in vitro* kinase assay these areas may be important for the conformational change needed to create an active kinase *in vivo*. To test whether these residues were important for conformational change, HEK293 cells expressing the mutated biosensor constructs were trypsinized and suspended in PBS for analysis in a spectrofluorometer. Two of the three mutations, F147C and D407V, caused no conformational change indicating no effect on the maintenance or release of the autoinhibitory conformation. Nor did they result in increased tyrosine phosphorylation when expressed transiently in cells, confirming the results from the *in vitro* kinase assay. The third, A590V mutant, exhibited an aberrant emission profile (Figure

3 A & B). The expression level of the mutant was low and exhibited a phosphorylation defect when the biosensor was transfected into HEK293 cells. This mutation is in the highly conserved “APE” sequence in the C-lobe of the kinase domain. As such, this sequence may be essential for the stability of the kinase domain. These results indicate that while FAK is a major player in cancer, these three naturally occurring mutations are not the mechanism by which its activity is increased. This underscores the importance of understanding the molecular structure and signaling pathways that regulate FAK autoinhibition.

Rational design of FERM domain mutations based on crystal structures

In order to identify residues that may be important for the physiological regulation of FAK activity, the available crystal structures of FAK were analyzed. Currently, there are two crystal structures available for the FAK FERM domain, the FERM domain alone (PDB: 2AL6) and the FERM domain in complex with the kinase domain (PDB: 2JoK) [10,14]. FERM F2 subdomain of FAK contains a basic patch that is necessary for FAK activation [13]. This basic patch consists of two ridges (Figure 4). The first ridge, ²¹⁶KAKTLRK²²¹, is on the apex of the F2 subdomain plays a role in FAK activation and some ligand binding [13]. The second, the RKK ridge is composed of R184/K190/K191, runs along the interface between the FERM domain and the Kinase domain. Examination of the two crystal structures for differences in FERM domain residues identified the RKK ridge as potential regulators of autoinhibition. In the FERM-catalytic domain crystal structure the side chains of these residues face the catalytic domain and the side chains interact with the side chains of residues on the kinase domain to form a salt bridge. Thus they could potentially be involved in inhibition through maintaining a charge-charge interaction with the kinase domain. In the structure containing FERM alone these side chains point away from the space that would be occupied by the kinase domain. This suggests the RKK ridge could play a role in activation through ligand binding to release the kinase domain. These candidates are supported by studies prior to the

crystallizations which mutated K190 and K191 and then observed a reduction in FERM domain binding to full length FAK [15]. However, the impact was largely unremarked upon because it was an intermediate effect overshadowed by the large change in FAK activity shown by mutating the ²¹⁶KAKTLRK²²¹ sequence [15]. Based on both the location of these residues and their altered positions in the crystal structures, it was hypothesized that these are key regulatory residues for autoinhibition (Figure 4B).

Impact of FERM Domain Residues on Basal Conformation

To determine the impact of R184 and K190 on activation of FAK, these residues were mutated to alanine in the FRET based CYFAK₄₁₃ biosensor [13]. HEK293 cells transfected with the wild type or mutated CYFAK₄₁₃ biosensor were held in suspension in a cuvette and FRET was measured utilizing a spectrofluorometer. FAK should be less active in nonadherent cells so this is an ideal way to observe activating mutations. When transfected into HEK293 cells, the mutated version of the biosensor had lower levels of FRET (Figure 5A) indicating the mutated FAK molecule has a more open conformation. This suggests these residues have a role in maintaining the autoinhibited conformation. This difference, while consistent, was not statistically significant (Figure 5B).

To assess the role of these residues in FAK in a more physiologically relevant context, HEK293 cells transfected with the CYFAK₄₁₃ biosensor were attached to tissue culture treated plastic 96-well plates in serum containing media. This allowed CFP, YFP, and FRET channel emissions to be observed using a Synergy H4 plate reader. Adhesion is important for the assessment of FAK conformation, as outside of focal adhesions FAK should always be inhibited. In adherent cells the R184A mutant had a FRET signal indistinguishable from wild type. While the K190A mutant had a trend towards a lower level of FRET these results were not statistically different from

wild type (Figure 5C). These results suggest the residues R184 and K190 might play a role in maintaining the autoinhibitory, closed conformation of FAK.

To evaluate the role of the entire ridge a triple-mutant was created. The new construct R184A/K190A/K191A, was tested in adherent cells. The R184A/K190A/K191A mutant consistently had a lower FRET profile than wild type. However, again this was not statistically significant (Figure 6). These results suggest these residues have a stabilizing effect on the autoinhibitory conformation, but are not vital for maintaining that conformation as other residues can compensate.

Impact of FERM domain on lipid binding

As the ²¹⁶KAKTLRK²²¹ sequence binds acidic ligands such as PI(4,5)P₂ and phosphotyrosine, it was hypothesized that the RKK ridge may also be required for ligand binding. To assess FERM domain binding to phospholipids, large unilamellar lipid vesicles composed of 60% phosphatidylcholine (PC) and 40% phosphatidylethanolamine (PE) were prepared as control vesicles and experimental vesicles containing 10% PI(4,5)P₂/50%PC/40%PE were made. GST-FERM domain fusion proteins were incubated with lipid vesicles for 1 hour on ice prior to vesicle sedimentation at 100,000 x g for 1 hour at 4°C. The amounts of fusion protein partitioning into the pellet (lipid bound) and supernatant (free) were determined by analyzing the two fractions by S.D.S-PAGE and Coomassie blue staining. In the absence of lipid vesicles, each protein was exclusively found in the supernatant (Figure 7). In the presence of PC/PE vesicles, a fraction of the GST-FERM domain fusion protein co-sedimented with the vesicles (10.1%). A larger fraction of the GST-FERM domain fusion protein (47.5%) co-sedimented with PI(4,5)P₂ containing vesicles (Figure 7A). To determine the role of the FERM domain basic residues in vesicle binding, two mutants ²¹⁶KAKTLRK²²² and R184A/K190A/K191A were analyzed for co-sedimentation with lipid vesicles. The ²¹⁶KAKTLRK²²² mutant FERM domain fusion protein was found in the supernatant in the absence of lipid vesicles. In the presence of PC/PE lipid vesicles, a fraction of the

²¹⁶KAKTLRK²²² fusion protein co-sedimented with the vesicles (10.8%). A similar fraction of the mutant fusion protein co-sedimented with PI(4,5)P₂-containing vesicles (15.6%) (Figure 7B). The R184A/K190A/K191A mutant FERM domain fusion protein was found in the supernatant in the absence of lipid vesicles. In the presence of PC/PE lipid vesicles, a fraction of the R184A/K190A/K191A fusion protein co-sedimented with the vesicles (21.5%). A larger fraction of the mutant fusion protein co-sedimented with PI(4,5)P₂-containing vesicles (37.0%) (Figure 7C). These findings demonstrate that the FAK FERM domain exhibits some binding to PC/PE vesicles, that binding is increased in the presence of PI(4,5)P₂. Additionally they confirm that the ²¹⁶KAKTLRK²²² ridge is the main binding site on the FERM domain. However mutation of the R184, K190, and K191 reduces the amount of PI(4,5)P₂ binding from 47.5% to 37.0% indicating that this may be a secondary phospholipid binding site.

Development of an Assay to Assess FAK Response to Activating Stimuli

Up until this point, FRET experiments were performed in unstimulated cells. For evaluation of the role of the basic ridge residues in response to activating stimuli, a new assay needed to be developed. This assay needed to enable the observation of relatively quick changes in FAK activation. While adhesion leads to a more active FAK population it is a slow process and poses imaging challenges as cells change shape over time. Several stimuli have been shown to activate FAK. Lysophosphatidic acid (LPA) stimulation and treatment with platelet derived growth factor were utilized previously to activate FAK [19]. Hepatocyte Growth Factor (HGF) or “scatter factor” binds to and activates the met receptor which in turn binds to and activates FAK [20]. This activation triggers cell motility and epithelial cells will take on a more mesenchymal phenotype [21]. The molecular mechanisms regulating FAK in response to HGF are better established than other stimuli. For activation by HGF the ²¹⁶KAKTLRK²²¹ sequence is needed. This makes it a better stimulus for experimentation as the KAKTLRK mutant can be utilized as a control. It is expected,

but not proven, that HGF stimulation leads to FAK conformation change, so experiments establishing this were performed. The CYFAK₄₁₃ biosensor was co-transfected with the Met receptor or tpr-met, an oncogenic fusion protein that leads to a constitutively active met kinase domain, into HeLa [22]. This co-expression lead to a decrease in FRET and a more active FAK population in suspended cells (Figure 7A). Subsequently, treatment of these cells with a met receptor inhibitor lead to a return to basal levels of FAK activation and FRET; confirming the FRET change was a direct result of met receptor activation (Figure 10B).

As they express the Met receptor and respond to HGF, as evidenced by morphology change and cell scattering Madin Darby Canine Kidney (MDCK) cells were transfected with the FAK biosensor. Using an epifluorescent microscope fitted with a live cell chamber, FRET signal was monitored to establish a baseline FRET ratio. FRET Ratio was defined as the emission in the CFP channel divided by the emission in the YFP channel when excited at the CFP excitation wavelength. Once a baseline was established cells were treated with 20ng/ul HGF and FRET measurements were acquired at 1 min intervals over a period of an hour. The FRET ratio at each of these time points is then compared to the baseline ratio established at the beginning of the experiment. The FRET ratio of wild type biosensor in untreated cells stayed constant over an hour of imaging. In cells treated with HGF, the FRET ratio increased over the course of imaging (Figure 7C). As this ratio is inverted an increase is indicative of decreasing YFP emission at CFP excitation, indicating a decrease in FRET and thus a release of autoinhibition in response to HGF stimulation.

Impact of FERM Domain Residues on Response to Activating Stimuli

The HGF assay can assess activation in response to ligand. The mutation of the RKK ridge residues may lead to destabilization of the autoinhibited conformation and sensitize activation. Additionally, If these residues are needed to bind ligand, this assay can detect an impairment in response to activating signals. Utilizing the HGF assay, MDCK cells transfected with the wild type

and mutated biosensors were monitored for FRET efficiency following stimulation. There was no difference in the rate of change in FRET in response to HGF stimulation over time between wild type biosensor and R184A/K190A mutant (Figure 10A). However the K190A/K191A mutant appeared sensitized to upstream signals with a greater change in FRET over time. Interestingly, the response from both of these mutants was much more varied than the wild type biosensor, as illustrated by the error bars (Figure 10A). To better assess the effect of this ridge the R184A/K190A/K191A triple mutant response to HGF stimulation was evaluated. While the mutation of this entire ridge did not yield a net change in FRET ratios statistically different from that of wild type, again the variance of response was much higher as illustrated by the error bars (Figure 10B). This indicates that while the net effect of mutating these residues is small, they may play dual roles in ligand binding and autoinhibitory conformation stabilization, which cancel their effects out.

Discussion

While the chromosomal region that contains the FAK gene (8q24.3) is susceptible to carcinogenic mutations, mutation of FAK in patient samples is rare [23]. In cancer over expression of the *fak* gene is the mechanism for altered FAK activity, and this overexpression of FAK is linked to poor outcomes especially in ovarian and breast carcinomas [24–26]. Our hypothesis was that the mutations identified in the COSMIC database contained in the FERM and catalytic domains of FAK, would result in activation of FAK. However, as our data shows, the F147C and D402V mutations do not affect the autoinhibition of FAK. The A590V mutation created a nonfunctioning version of the biosensor so its effect on autoinhibition could not be properly assessed. It did however result in a kinase dead mutant. A590 is in the highly conserved APE sequence that is considered the “end” of the activation loop, so might cause a change in activation loop structure upon phosphorylation. The FAK effect in the A590V mutant is potentially due to its scaffolding capabilities. Thus the FAK effect in these cancers must be a result of different mechanisms. This evidence emphasizes the importance of understanding the signaling pathways that activate FAK and the mechanism for FAK overexpression in cancer.

The second set of residues investigated in this study were identified through investigation of the crystal structures of FAK. These residues form one of two basic ridges along the F2 subdomain of FAK. The first ridge, KAKTLRK, binds to PIP₂ and is necessary for proper FAK activation [15,16]. The second ridge formed, by R184, K190, and K191, is part of the interface between the FERM and kinase domains in the autoinhibited structure [10]. They also border the pocket that holds F596 in the autoinhibited conformation [14,16]. Besides the location of these residues the positions of their side chains in crystal structures were intriguing. In the crystal structure of the FERM domain alone the side chains are pointed up and away from the space that would be occupied by the catalytic domain [10]. The crystal structure containing both the FERM and kinase domains,

the side chains of these residues are facing the catalytic domain [14]. This change in side chain position suggested a switch in positioning between the autoinhibited and active conformation. Interestingly, a K190AK191A mutant was investigated during the characterization of KAKTLRK [15]. While not thoroughly investigated, a GST-KK construct had reduced pull down of full length FAK suggesting a reduction in the maintenance of the autoinhibited conformation [15].

Mutation of these basic residues to alanine had no effect on FAK catalytic activity but it did have some effect in the FRET biosensor signal. The decrease in FRET signal, while not significant was consistent. These results suggest that while the basic side chains are not essential for the autoinhibited conformation they may be important for stabilization. The lack of a difference in FRET change in response to upstream activating signals suggests that these residues are not important for binding a negative ligand like KAKTLRK. However, lipid sedimentation studies with the RKK mutant lead to a 10% decrease in lipid binding. This affinity may be masked as these residues are partially buried in the autoinhibited conformation. Together these experiments expand the picture of FAK autoinhibition, in which the side chains of the FERM domain help to stabilize the conformation held together by the lock and key created by Y180, M183, and F596.

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stabilize the conformation held together by the lock and key created by Y180, M183, and F596. The original hypothesis posed was that the RKK ridge shifts conformation during ligand binding to release the catalytic domain. These studies suggest that autoinhibition is released through another mechanism. Based on the current literature, the most plausible hypotheses are a mechanosensory mechanism in which cytoskeletal force pulls the two domains apart, or binding of ligands to a second site on FAK to prompt release of the autoinhibited conformation.

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Figure Legends

Figure 1: The FERM-kinase autoinhibited structure. The FERM domain is shown in light magenta and the kinase domain is shown in light cyan. The linker is shown in grey. Side chains of residues that were mutated are shown as sticks.

Figure 2: Biochemical characterization of COSMIC mutants A) Western blot showing FAK expression levels. B) Western blot showing the pY397 levels from cells transfected with wild type or mutated FAK. (n = 3) C) FAK western blot showing the amount of FAK contained in an *in vitro* kinase assay. D) Western blot showing the amount of phosphorylated substrate at the end of an *in vitro* kinase assay. (n = 3)

Figure 3: Conformational characterization of COSMIC mutants A) FRET ratios for wild type and mutated biosensors (n = 12). B) A representative emission scan of wild type and mutated biosensors.

Figure 4: Structural features of interest in the FERM domain A) A surface view of the FERM-kinase domain autoinhibited structure. The FERM domain is shown in light magenta, the catalytic domain is shown in light cyan. The KAKTLRK ridge is shown in purple and the RKK ridge is shown in magenta. B) A ribbon diagram of the FERM-catalytic domain crystal structure. The side chains of R184, K190, and K191 are shown as sticks. C) A ribbon diagram of the FERM domain crystal structure. The side chains of R184, K190, and K191 are shown as sticks.

Figure 5: Conformational characterization of R184A and K190A in suspended cells A) A representative emission scan of wild type and mutated biosensors. B) FRET Ratios of wild type and mutated biosensors in suspended cells (n = 12). C) FRET ratios of wild type and mutated biosensors in adherent cells (n = 40).

Figure 6: Conformational characterization of R184A/K190A/K191A in adherent cells A) A representative emission scan of wild type and mutated biosensors. B) FRET Ratios of wild type and mutated biosensors in adherent cells (n = 10).

Figure 7: R184A/K190A/K191A reduces FERM lipid binding. GST fusion proteins were incubated in buffer alone or with large unilamellar vesicles comprised of 60%PC/40%PE (PC/PE) or 50%PC/40%PE/10% PI(4,5)P₂ (PI(4,5)P₂). The samples were sedimented at 100,000 x g and the supernatant (S) and pellet (P) fractions analyzed by S.D.S-PAGE and Coomassie blue staining (Top Panel A, B, and C). The results of quantification of multiple experiments ($n=3$) is shown in the bottom panels. The partitioning of the wild type FERM domain of FAK (panel A), the KAKTLRK mutant FERM domain (Panel B) and the RKK mutant FERM domain (panel C) between supernatant (S) and pellet (P) fractions is shown. Gels were analyzed using ImageJ and the fraction of each protein in the supernatant and pellet (+/- S.D.) was plotted.

Figure 8: Development of an assay for FAK response to upstream signals A) A representative emission scan of cells transfected with either biosensor (413) alone or with wild type Met or TPR-Met. B) A representative emission scan of cells transfected with either biosensor (413) alone or with wild type Met and treated with a met receptor inhibitor. C) Comparison of FRET ratio over time between untreated cells and cells treated with 20ng/ul of HGF ($n=6$).

Figure 9: R184A/K190A/K191A does not affect FAK response to activating signals. A) The change in FRET ratios over time for wild type and mutant biosensor transfected MDCKs treated with HGF ($n = 28-32$ cells each over 4 experiments). B) The change in FRET ratios over time for wild type and mutant biosensor transfected MDCKs treated with HGF ($n = 40 - 58$ cells each over 6 experiments).

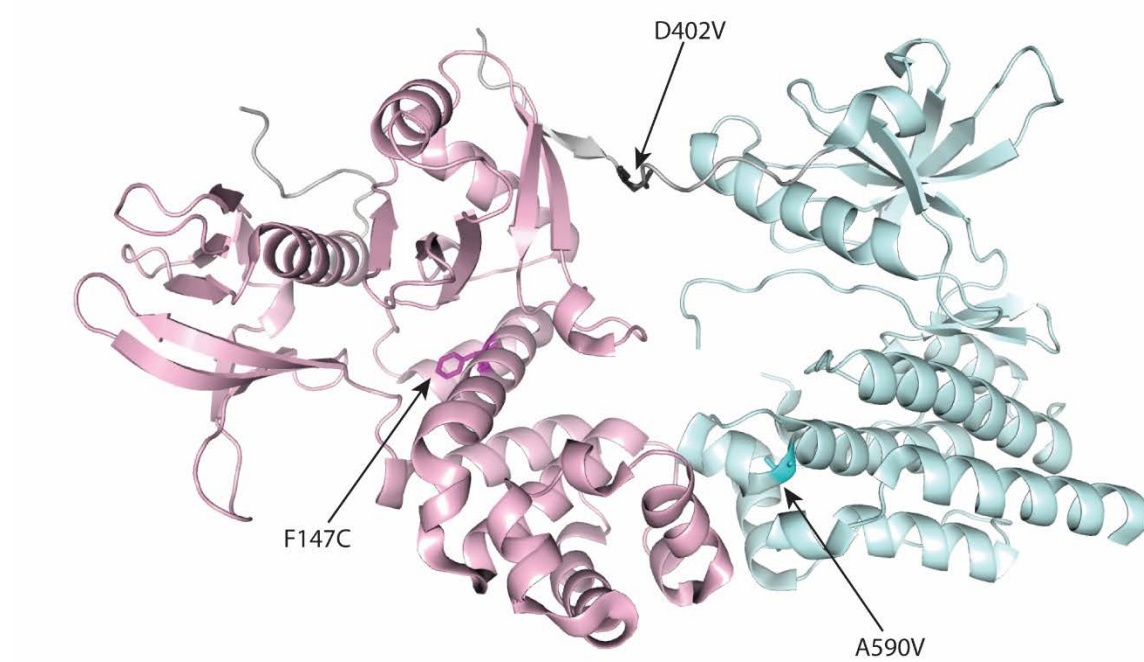


Figure 1

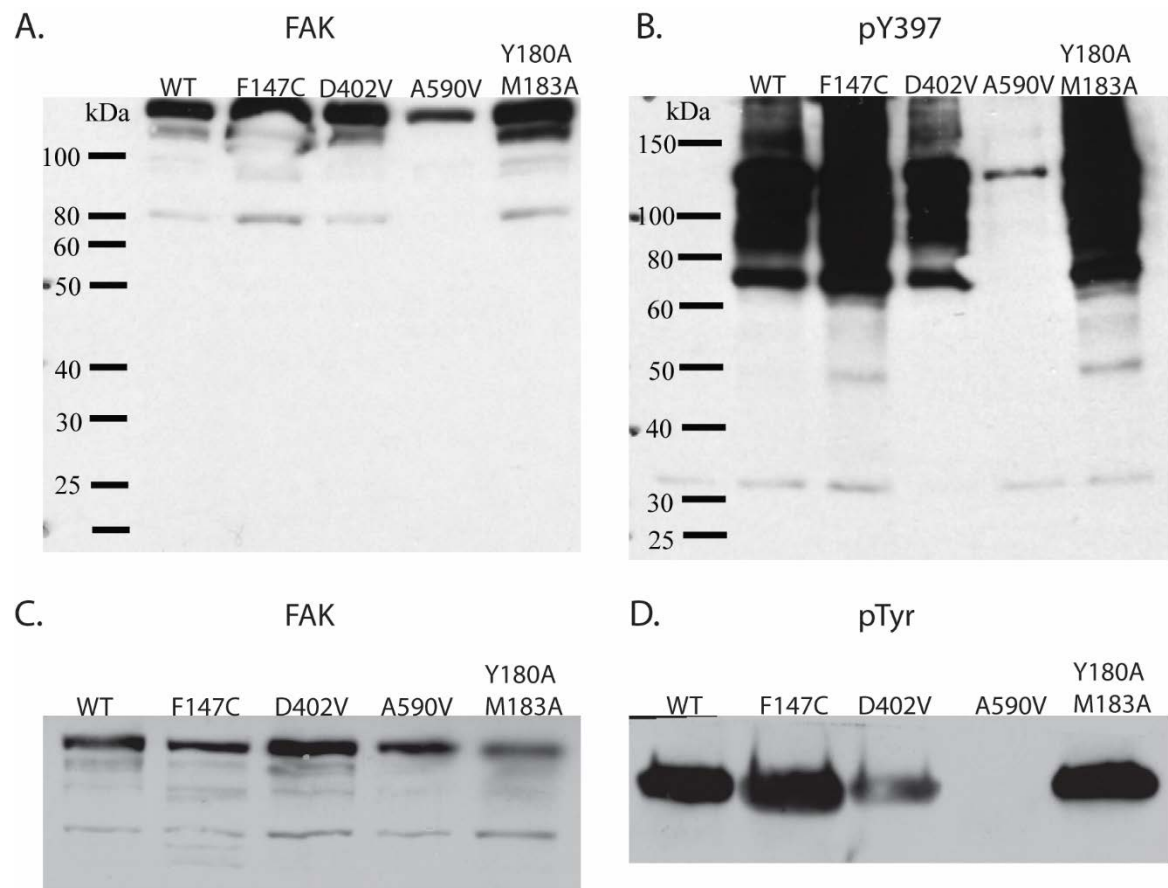
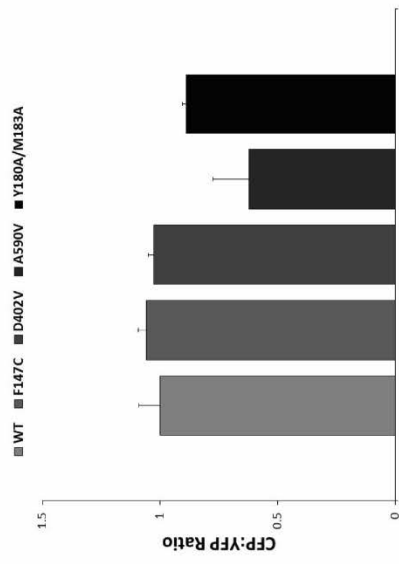


Figure 2

A.



B.

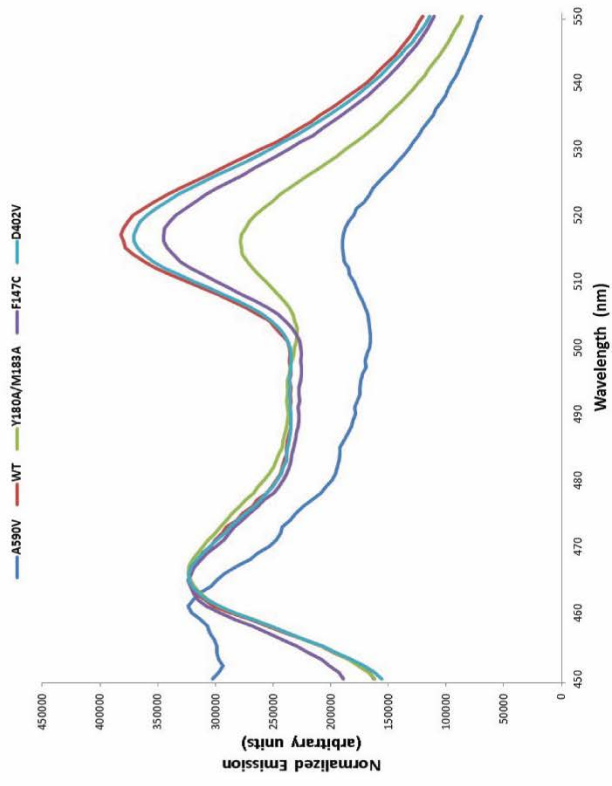


Figure 3

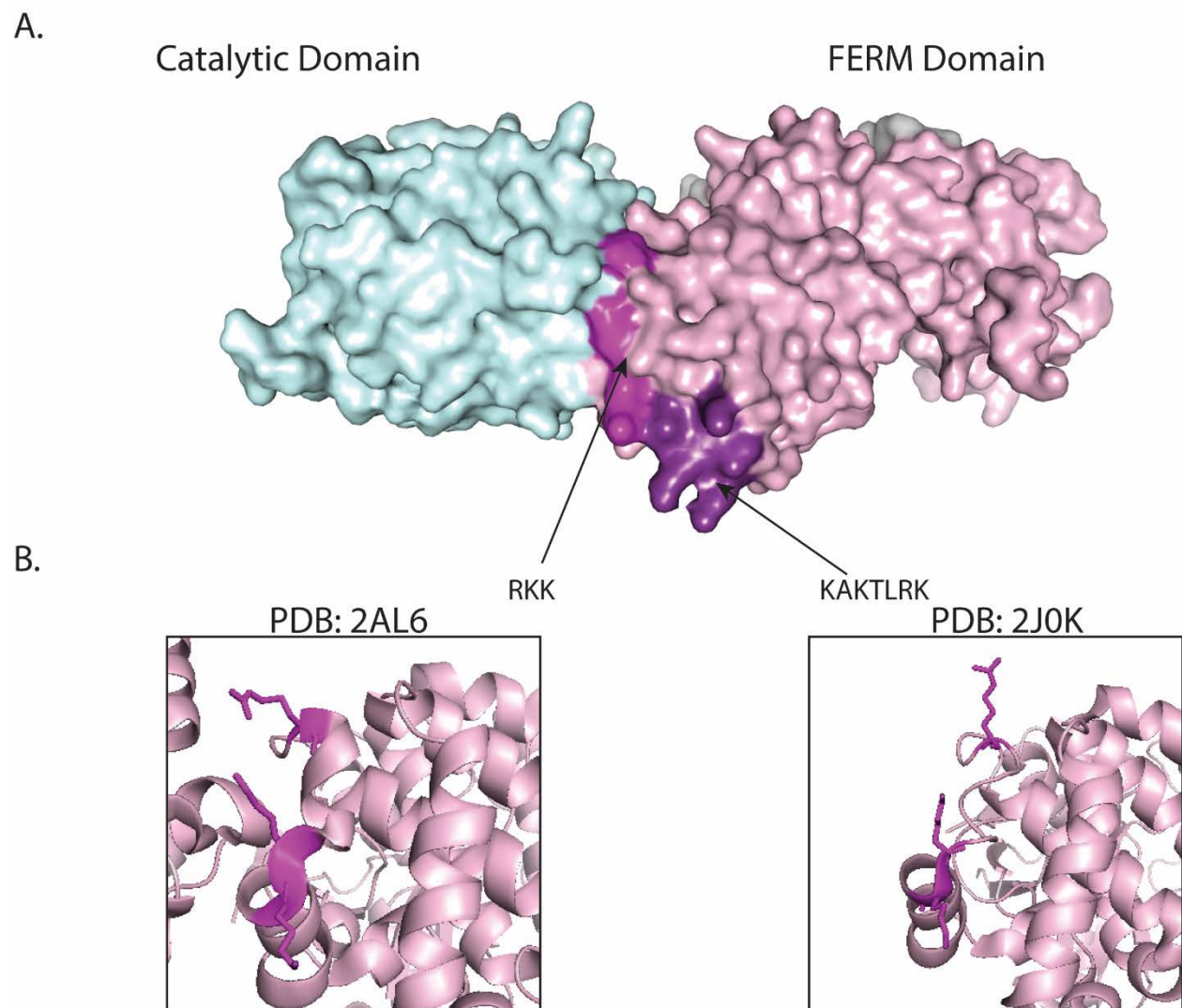
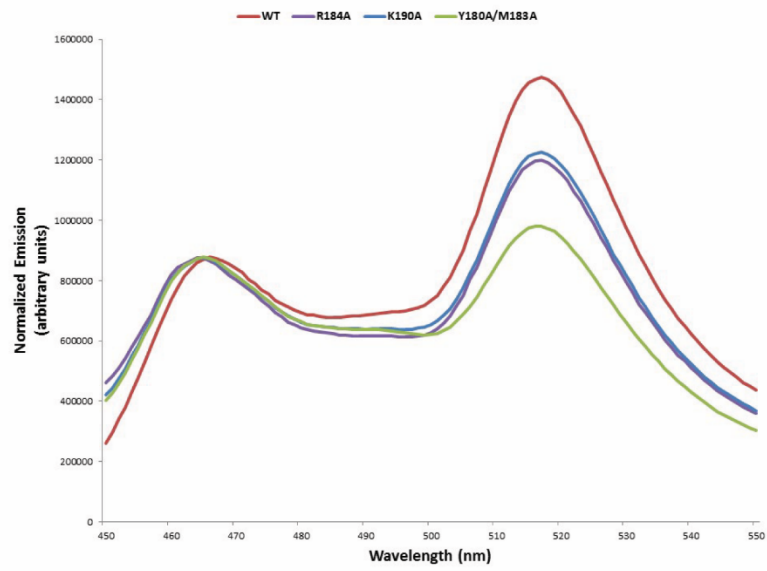
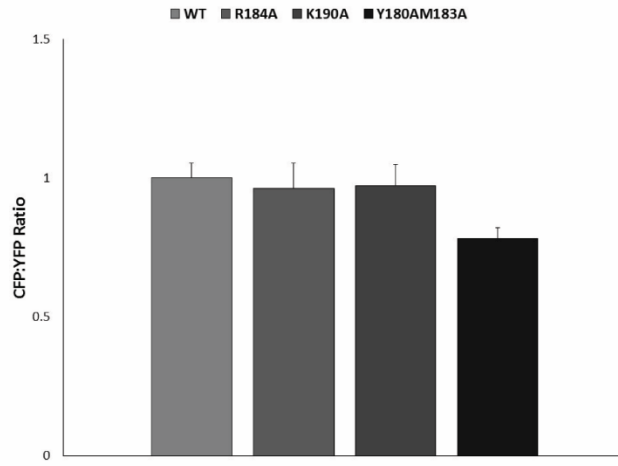


Figure 4

A.



B.



C.

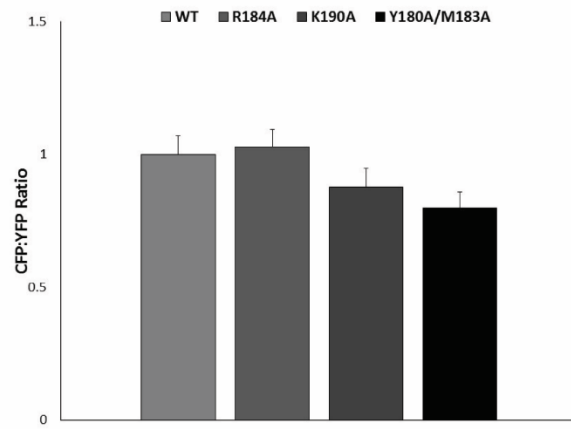
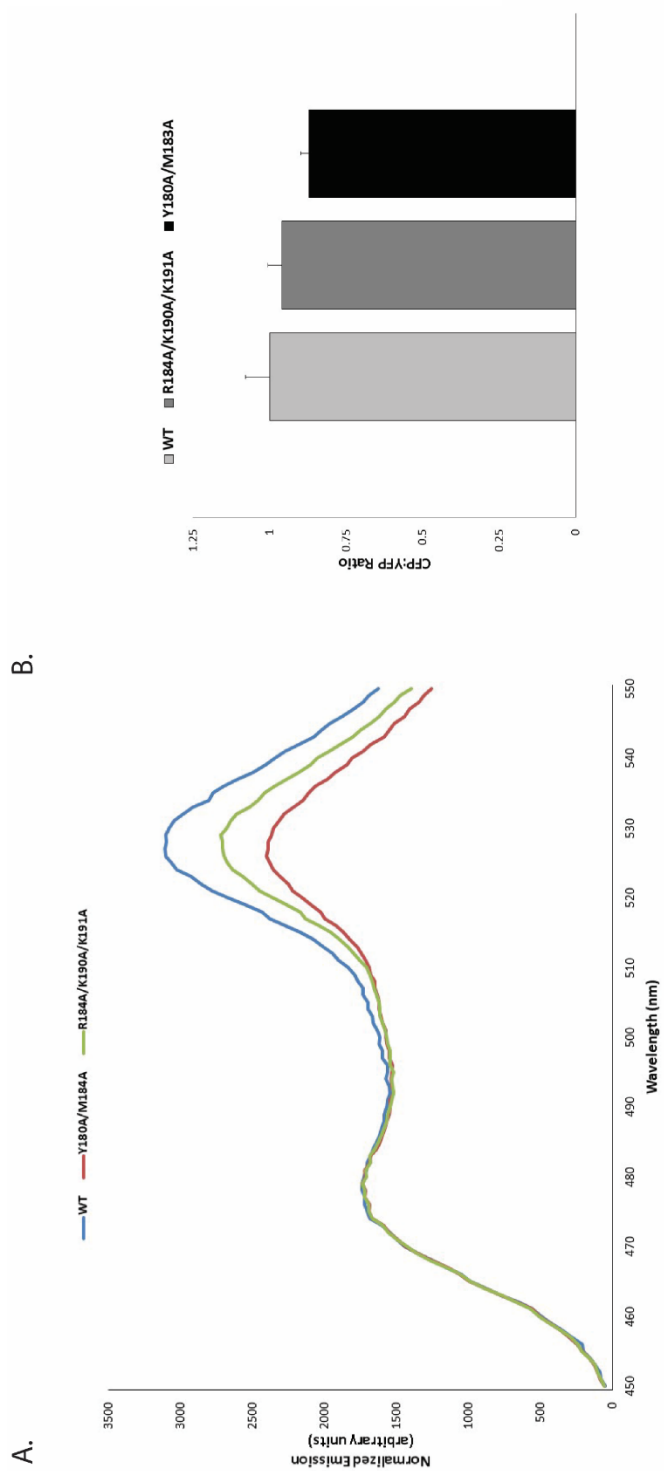


Figure 5

Figure 6



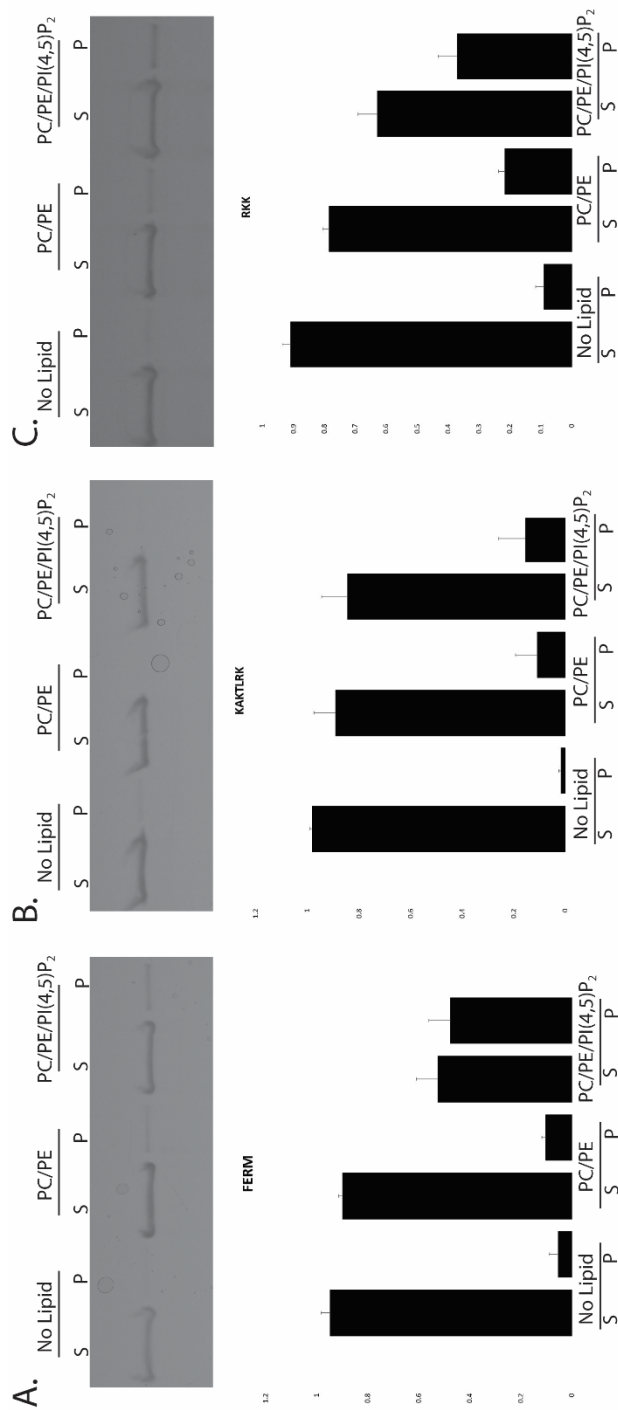
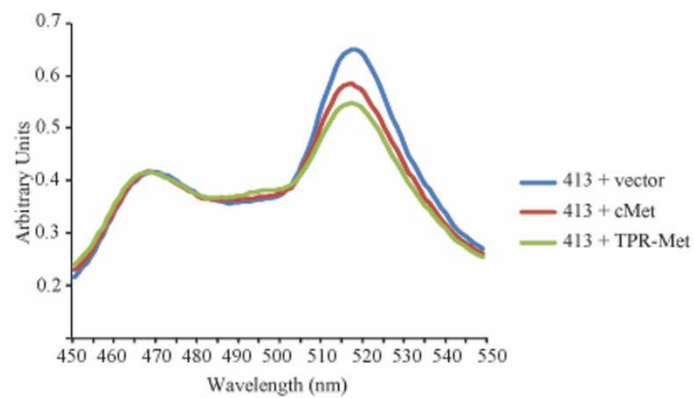
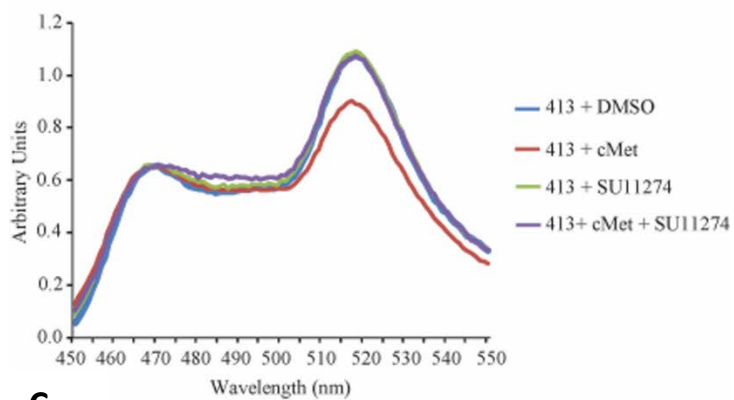


Figure 7

A.



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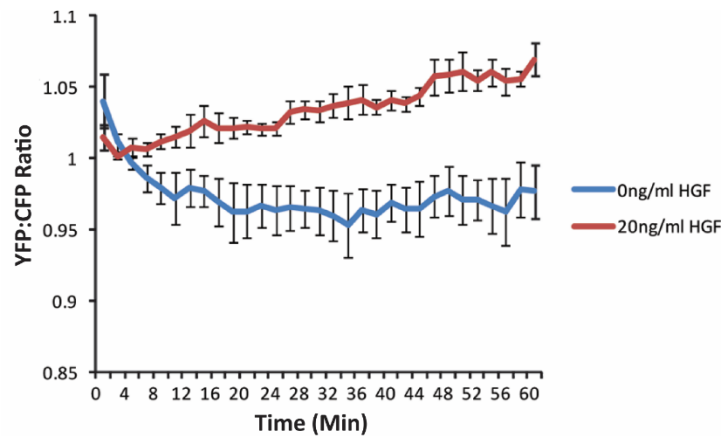


Figure 8

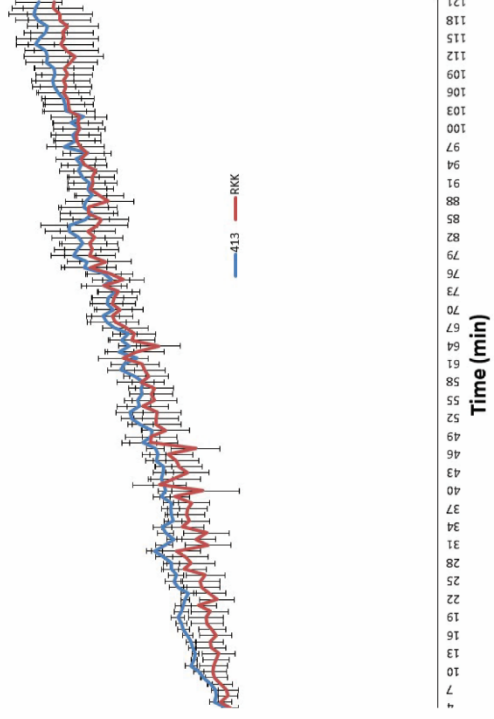
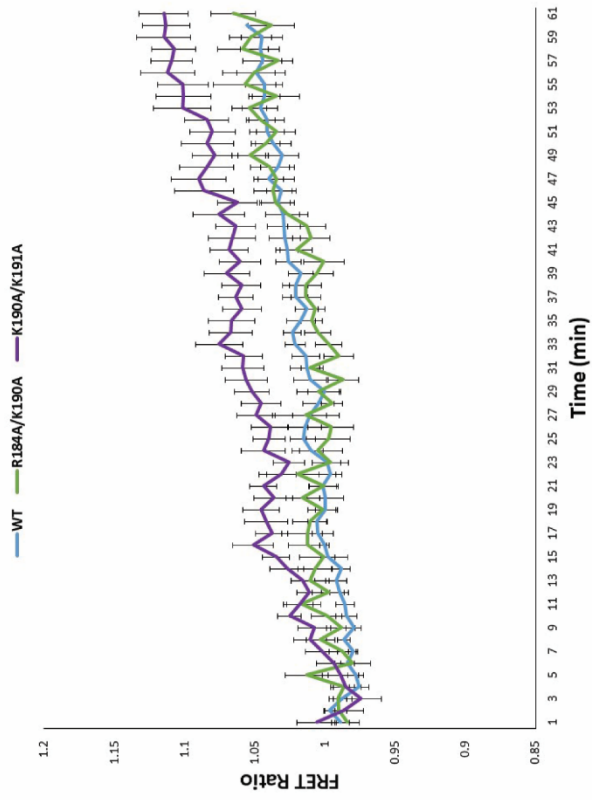


Figure 9

Chapter 3: Phospholipid Binding to the FAK Catalytic Domain Impacts Function

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Abstract

Focal adhesion kinase is an essential nonreceptor tyrosine kinase that plays an important role in development, in homeostasis and in the progression of human disease. Multiple stimuli activate FAK, which requires a change in structure from an autoinhibited to activated conformation. In the autoinhibited conformation the FERM domain associates with the catalytic domain of FAK and PI(4,5)P₂ binding to the FERM domain plays a role in the release of autoinhibition, activating the enzyme. An in silico model of FAK/PI(4,5)P₂ interaction suggests that residues on the catalytic domain interact with PI(4,5)P₂, in addition to the known FERM domain PI(4,5)P₂ binding site. This study was undertaken to test the significance of this in silico observation. Mutations designed to disrupt the putative PI(4,5)P₂ binding site were engineered into FAK. These mutants exhibited defects in phosphorylation and failed to completely rescue the phenotype associated with *fak*^{-/-} phenotype fibroblasts demonstrating the importance of these residues in FAK function. The catalytic domain of FAK exhibited PI(4,5)P₂ binding in vitro and binding activity was lost upon mutation of putative PI(4,5)P₂ binding site basic residues. However, binding was not selective for PI(4,5)P₂, and the catalytic domain bound to several phosphatidylinositol phosphorylation variants. The mutant exhibiting the most severe biological defect was defective for phosphatidylinositol phosphate binding, supporting the model that catalytic domain phospholipid binding is important for biochemical and biological function.

Introduction

Focal adhesion kinase (FAK) is an essential non-receptor tyrosine kinase since *fak*^{-/-} mice exhibit embryonic lethality. FAK is broadly expressed in different cell types and loss of FAK expression or function results in multiple embryonic defects including deficits in angiogenesis, formation of the neural tube and development of a multi-chambered heart [1–3]. Homeostasis in some adult tissues is also dependent upon FAK, as demonstrated in conditional FAK knockout mouse models. For example, in keratinocytes FAK is necessary for maintenance of an epidermis of normal thickness and for sebaceous gland function, and in intestinal epithelial cells, FAK is required for efficient mucosal wound healing [4,5]. FAK also plays a role in the development of pathologies associated with several human diseases. The most extensive evidence has implicated FAK in the development and progression of a number of cancers and significant efforts to therapeutically target FAK with small ATP analog inhibitors have been made [6,7]. FAK has also been implicated in atherosclerosis, and the tissue remodeling that occurs during cardiac hypertrophy [8–10]. At the cellular level, FAK regulates cell proliferation, cell survival, and cell migration, and control of these cellular events underpins the biological functions of FAK in development, homeostasis, and disease [11–13].

Given its broad distribution of expression across tissues and role in controlling multiple cellular and biological functions, it is not surprising that a wide range of stimuli can activate FAK signaling. Integrin-dependent cell adhesion to extracellular matrix proteins is a major activating signal and soluble ligands for receptor tyrosine kinases and G protein coupled receptors can trigger FAK activation [14]. These diverse stimuli initiate catalytic activation of FAK and autophosphorylation, which creates a binding site for Src, another nonreceptor tyrosine kinase, which in turn phosphorylates FAK at additional sites [15–17]. Tyrosine phosphorylation of FAK stimulates maximal catalytic activity in addition to regulating FAK's ability to serve as a scaffold to assemble a complex of signaling molecules to transduce downstream signals [18].

The critical event in FAK activation is a conformation change from an autoinhibited to an activated state. In the autoinhibited conformation, the FAK band 4.1, Ezrin, Radixin, Moesin (FERM) domain binds to the catalytic domain to occlude the active site and substrate binding site[19]. There are two contact sites in this conformation, one between the C-terminal lobe of the kinase domain and the F2 lobe of the FERM domain and the second between the N-terminal lobe of the kinase domain, the linker between the FERM and kinase domains, and the F1 lobe of the FERM domain (Figure 1). Activating stimuli are proposed to modify the FERM domain or generate a FERM domain binding ligand that reduces the affinity of the FERM domain for the catalytic domain relieving the inhibitory interaction and/or stabilizing the active conformation. Phosphorylation of FAK at tyrosine 194 by the Met receptor tyrosine kinase is proposed to relieve autoinhibition in response to hepatocyte growth factor stimulation, and Src-dependent phosphorylation of the activation loop of FAK is believed to sterically block FERM domain binding to the catalytic domain, thus stabilizing the active conformation [19,20]. A change in intracellular pH also modulates FAK activity through a mechanism of protonation/deprotonation of histidine 75, which resides in the FERM domain and is proposed to alter the stability of the autoinhibited conformation [21]. A FERM domain basic sequence in the F2 lobe lies near the interface with the kinase domain and is required for FAK activation [22]. This sequence serves as a binding site for two different ligands, a tyrosine phosphorylated peptide from the Met receptor and PI(4,5)P₂ [23,24]. Binding of these ligands to the basic sequence in the FERM domain is envisioned to relieve autoinhibition.

The role of PI(4,5)P₂ binding in FAK activation has been experimentally addressed using biochemical and computational approaches. FAK binding to PI(4,5)P₂ is cooperative and induces FAK clustering *in vitro* [25]. The FERM domain mediates FAK dimerization, which is required for biochemical and biological function *in vivo* [26]. However, dimerization occurs *in vitro* in the

absence of PI(4,5)P₂ and thus the role of this interaction in PI(4,5)P₂-induced clustering has not been elucidated. Molecular dynamics simulations have been performed to gain insight into the molecular mechanism of FAK activation by PI(4,5)P₂. Allosteric connectivity is observed between alpha helix C and alpha helix G, which reside in the N-terminal and C-terminal lobes of the catalytic domain respectively. Alpha helix C contacts the linker, which contacts the F₁ lobe of the FERM domain, and alpha helix G directly contacts the F₂ lobe of the FERM domain in the autoinhibited state [19,25]. PI(4,5)P₂ docking induces local changes in the F₂ lobe of the FERM domain, and also induces long range changes between the N-terminal lobe of the kinase domain and the F₁ lobe of the FERM domain [25,27]. In these simulations, PI(4,5)P₂ binding induced small changes in the conformation of the FERM/kinase domain and did not cause dissociation of the two domains [25,27,28]. Additional events may therefore contribute to the conversion of the autoinhibited to fully activated state. Molecular dynamics simulations have demonstrated that PI(4,5)P₂ sufficiently tethers the FERM domain to allow mechanical separation of the FERM and catalytic domains when forces are applied to the C-terminus of the catalytic domain [29]. This is an interesting model given FAK's role in sensing mechanical stiffness of the extracellular matrix and the requirement of an intact actin cytoskeleton for activation [30–32]. Interestingly, one of the molecular dynamics simulation studies suggested a novel interaction between the catalytic domain and PI(4,5)P₂ [28]. In addition to the interaction of the F₂ lobe basic sequence with PI(4,5)P₂, basic residues in the catalytic domain also docked with PI(4,5)P₂ in these simulations. Of interest were a number of basic residues on the side of the large lobe of the catalytic domain as a rotation of the catalytic domain with respect to the FERM domain might be required for membrane binding, and this movement could potentially contribute mechanistically to FAK activation. The current study was undertaken to test the biochemical and biological significance of this observation.

Materials and Methods

Molecular Biology

FAK mutants were created using a modified version of the Quickchange (Agilent) mutagenesis protocol using the viral vector pLUdr containing wild type avian FAK cDNA or the expression vector pGEX-KG containing wild type avian FAK catalytic domain as a template. Primers for site-directed mutagenesis were designed to substitute alanine for lysine or arginine residues at the sites of interest. Sequence analysis verified the presence of the intended mutations and that no unintended mutations were present. (ACGT Inc.).

Cell Culture and Imaging

Human embryonic kidney (HEK) 293T cells and mouse embryonic fibroblasts (MEFs) (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Streptomycin/Penicillin. HEK 293T cells were co-transfected with the pLUdr-FAK vectors and the PAX2 and VSVG viral packaging vectors using Turbofect (Thermo Fisher) according to the manufacturer's instructions. Media containing virus was collected at 24, 48, and 72 hours. Media was pooled and filtered using a 0.45 μ m filter (Milipore). *fak*^{-/-} MEFs (ATCC) were incubated with the resulting virus-containing supernatant for 6 hours and selected with puromycin after 24 hours. Puromycin selection was carried out for five days.

For immunofluorescence experiments, cells were plated on fibronectin coated coverglass overnight. Cells were fixed in 3% formaldehyde for 15 min and permeabilized with 0.4% Triton X-100 for 10 min. FAK was detected using the BC4 anti-FAK antibody and an Alexa Fluor488 (Invitrogen) conjugated anti-rabbit secondary antibody as previously described [22]. Paxillin was detected using an anti-paxillin antibody (BD Biosciences) and an Alexa Fluor 488 conjugated anti-mouse secondary antibody (Invitrogen). Cells were visualized by using a Zeiss Fluorescent Axio Imager Z2 microscope with Zeiss 63x (N.A. 1.40) objective. Images were taken with an AxioCam

MRm Rev.3 camera with identical exposure times. Images were analyzed using ImageJ. Individual cells were selected and the contrast inverted. The threshold of this overlay was set to restrict analysis to focal adhesions. The area of focal adhesions was calculated using the Analyze Particles function. The scale was set so that pixel area was converted into μM^2 .

To measure cell spreading, *fak*^{-/-} cells expressing wild type or mutant FAK were transfected with GFP-paxillin to allow live cell imaging. Cells were plated on fibronectin coated imaging glass dishes. After a five minute incubation period to allow attachment, dishes were placed in an environmental chamber mounted onto a Nikon Swept Field confocal microscope. Cells expressing GFP-paxillin were visualized using a Nikon 40x oil objective and images were taken at one minute intervals with an exposure time of 200 msec for GFP. Background subtraction was performed. Cells were outlined and cell diameters at each time point were recorded using the Nikon NIS Elements analysis software.

To measure focal adhesion dynamics *fak*^{-/-} cells expressing wild type or mutant FAK were transfected with GFP-paxillin to allow identification of adhesions. Cells were placed on fibronectin coated imaging glass dishes for 4 hours to allow for spreading defects between cell lines. Cells were refed with imaging media and placed in an environmental chamber mounted on a Nikon Swept Field confocal microscope. Cells were visualized utilizing a Nikon 60x oil objective and images were taken at one minute intervals with an exposure time of 200 msec over an hour. Background subtraction was performed. Focal adhesions were analyzed utilizing the Focal Adhesion Analysis Server [33,34]. This server allows the log-linear fitting method determination of assembly and disassembly phase length in which models are fit to all assembly and disassembly phases greater than or equal to a length of 2 minutes, as described in Webb et al. [35].

Protein Analysis

Cells were lysed in ice-cold modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.3], 150 mM NaCl, 1% IGEPAL, 1% Nonidet P-40, 0.5% deoxycholate, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM vanadate). Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid assay (Pierce). Lysates were boiled in Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% S.D.S, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) and 15 μ g of total protein was analyzed by Western blotting. An anti-FAK antibody (4.47 - Millipore), an anti-phosphotyrosine antibody (4G10 - Millipore) and anti-FAK phosphospecific antibodies (PTyr397, PTyr577 and PTyr861 - Invitrogen) were used as primary antibodies for Western blotting. Horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies (Millipore) were used to detect FAK expression and phosphorylation levels using Immobilon Western HRP chemiluminescence substrate (Millipore). Quantification of blots was performed with ImageJ (<http://rsb.info.nih.gov/nih-image>). Developed films were scanned as TIFF images in 8-bit grayscale format at 600 dpi. The lanes were defined using the rectangular select tool and the Analyze \rightarrow Gels \rightarrow Select First Lane/Select Next Lane function. Densitometry measurements were calculated using Analyze \rightarrow Gels \rightarrow Plot lanes. Band peak was defined from background using the straight line tool then the area of the band peak was calculated using the Wand tool. (Protocol can be found at <http://rsb.info.nih.gov/nih-image/manual/tech.html#analyze>.) Band intensities of the phosphotyrosine blots were divided by the band intensities of the FAK blots and all wild type samples were normalized to one.

Protein purification

The expression of recombinant protein in *Escherichia coli* BL21 (Codon Plus) cells (Agilent) was induced at an absorbance (OD) at 600 nm of 0.8 to 1.0 by the addition of 0.1 mM IPTG (isopropyl- β -d-thiogalactopyranoside), and cells were grown for an additional 8 h at 22°C. The cells

were harvested and frozen at -20°C . Cell pellets were thawed and resuspended in lysis buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 4% Triton X-100, 10% sarkosyl, 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). Lysis buffer was supplemented with the addition of protease inhibitors at a final concentration of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10 $\mu\text{g/ml}$ of leupeptin, 10 mg/ml of lysozyme, and 1 mg/ml of DNase. The resuspended cells were lysed by sonication on ice. After clarification, supernatants were loaded onto a glutathione sepharose column. Following extensive washing with Tris buffered saline (pH 7.4), protein was eluted with a buffer containing 50 mM Tris [pH 8.0], 150 mM NaCl and 50 mM glutathione. Elution fractions were concentrated using Ultra-4 centrifugal filter units (10 kDa NMWL) (Amicon).

Lipid Binding

Lipid binding was assessed by co-sedimentation with large, unilamellar vesicles (LUV). The ability of the catalytic domain to bind $\text{PI}(4,5)\text{P}_2$ was analyzed using lipid vesicles containing 50%PC/40%PE/10% $\text{PI}(4,5)\text{P}_2$ (experimental) or 60%PC/40%PE (control). Vesicles were prepared by mixing chloroform dissolved phospholipids (Echelon) in appropriate ratios. The mixture was dried using a speed vacuum for 15 minutes. The dried lipid cake was suspended into a lipid binding buffer (20 mM HEPES [pH 7.5], 2 mM dithiothreitol, 250 mM NaCl) to a final concentration of 2.5 $\mu\text{g/ul}$ [36]. The lipid suspension containing large multilamellar vesicles (LMV) was passed eleven times through a 100 nm filter using an Avanti mini-extruder set to ensure a homogenous suspension of LUVs. Four micrograms of glutathione S-transferase (GST) fusion protein was incubated with 250 μg of lipid vesicles on ice for 1h. The mixtures were centrifuged at $100,000 \times g$ for 1h at 4°C . The supernatants were collected, mixed with Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% S.D.S, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) and boiled. The pellets were resuspended in Laemmli sample buffer and boiled. The samples were analyzed by S.D.S-

polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. Quantification of gels was performed with ImageJ (<http://rsb.info.nih.gov/nih-image>). Images of gels were converted to TIFF images in 8-bit grayscale format at 600 dpi. The lanes were defined using the rectangular select tool and the Analyze → Gels → Select First Lane/Select Next Lane function. Densitometry measurements were calculated using Analyze → Gels → Plot lanes. Band peak was defined from background using the straight line tool, then the area of the band peak was calculated using the Wand tool. (Protocol can be found at <http://rsb.info.nih.gov/nih-image/manual/tech.html#analyze>.) Band intensities of the supernatant and pellet lanes were divided by the sum of the band intensities of the supernatant and pellet.

For anisotropy experiments purified GST fusion proteins were incubated with BODIPY (dipyrromethene boron difluoride)-labeled phosphoinositides with C6-acyl chains (Echelon). Increasing concentrations of purified protein were added to 12.5 nM fluorescent phosphoinositide in buffer containing 20 mM HEPES [pH7.5], 150 mM NaCl, and 5 mM β -mercaptoethanol. Anisotropy measurements were taken at 37°C using a Flourlog spectrofluorometer equipped with Glan-Thompson polarizers with FluorEssence software (HORIBA Jobin Yvon). Specific binding of the catalytic domain was determined by subtraction of binding of GST alone. Binding curves and dissociation constants were determined using the Prism 5.0 statistical analysis software (GraphPad).

Kinase Assay

White low-volume 96-well polystyrene plates (PerkinElmer) were used for the ADP-Glo assay. Four μ g of kinase was mixed with 25 μ L of kinase buffer (5 mM MgCl₂, 5 mM MnCl, 50 mM TrisHCl pH 7.4), 2.5 μ g Poly-Glu-Tyr, and 25 mM ultrapure ATP (Promega). All reactions were carried out in triplicate. Blank wells lacked enzyme but did include kinase buffer, substrate, and ATP. The plates were covered and the reactions were carried out at room temperature (RT) for up

to 120 min. Reactions were stopped with the addition of 50 μ L ADP-Glo reagent (Promega). After a 40-min incubation at RT, 50 μ L of Kinase Detection Reagent (Promega) was added and the plates were incubated for another 40 min at RT. Plates were read on a BioTek Synergy 4 plate reader with a sensitivity of 150 and an integration time of 1 s per well. For kinase reactions involving lipids, 8 μ g of kinase was preincubated with 25 μ L of lipid vesicle solution at RT prior to addition of 25 μ L of kinase buffer with 5 μ g poly-Glu-Tyr, 25 mM ATP. Data were plotted using Prism 5.0 statistical analysis software (GraphPad).

Results

Catalytic Domain Residues are Required for Maximal FAK Phosphorylation in vivo.

To assess the physiological relevance of the catalytic domain basic residues implicated in phospholipid binding by the molecular modeling simulations, select residues were substituted with alanine and engineered in the full length FAK cDNA in the pLUdr lentiviral vector (Figure 1). Since Arg508, Arg514 and Lys515 are located in a single alpha helix (α D), a mutant containing alanine substitutions for these residues was engineered (R508A/R514A/K515A). As Lys621 and Lys627 are located within the same loop between α F and α G of the FAK catalytic domain, another mutant with alanines substituted for these two residues was created (K621A/K627A). Finally, since Arg508 and Lys621 are in close proximity in the three-dimensional structure of the catalytic domain, a mutant with alanine replacing these two residues was created (R508A/K621A) (Figure 1B). The R508A/R514A/K515A, R508A/K621A, and K621A/K627A mutants were expressed in *fak*^{-/-} mouse embryo fibroblasts (MEFs). Populations of MEFs expressing each construct were lysed and lysates were analyzed by Western blotting for FAK expression and FAK phosphorylation (Figure 2A). Tyrosine phosphorylation levels were normalized to FAK expression levels and compared to WT FAK phosphorylation levels. FAK phosphorylation at Tyr397 and Tyr577 was significantly decreased in the R508A/K621A and K621A/K627A mutants (Figure 2 B&C). The R508A/R514A/K515A mutant exhibited a reproducible reduction in tyrosine phosphorylation at these sites, but the decrease did not reach statistical significance. All three mutants showed a reproducible reduction in phosphorylation at the Tyr861 site, with the R508A/K621A and R508A/R514A/K515A mutants showing a significant decrease (Figure 2 D). Autophosphorylation at Tyr397 is one of the most well defined steps in FAK activation and is necessary for Src binding and subsequent phosphorylation of Tyr577 and Tyr861. The reduction in phosphorylation of these sites is indicative of reduced FAK activation and demonstrates that these mutants are less responsive to upstream signals.

As localization of FAK is required for correct regulation, the subcellular localization of the FAK mutants was assessed by immunofluorescence to ensure the phosphorylation defects seen were not due to mislocalization. The *fak*^{-/-} MEFs exhibited no FAK staining (data not shown), while cells re-expressing wild type or mutant FAK exhibited staining with the anti-FAK antibody. The FAK mutants localized to focal adhesions comparably to wild type (Figure 3). This indicates that the reduced phosphorylation level of the FAK mutants is not attributable to poor localization.

Catalytic Domain Mutants Exhibit Impaired Biological Function

Knockout of FAK from cells leads to a variety of phenotypes associated with defects in focal adhesion turnover, including an increase in focal adhesion size and decreased cell spreading. To test the biological effectiveness of the catalytic domain mutants, focal adhesion size, focal adhesion dynamics and cell spreading were measured.

fak^{-/-} MEFs exhibit an increase in focal adhesion size and re-expression of wild type FAK rescues this phenotype [37]. To determine if the catalytic domain mutants were capable of rescuing this phenotype, cells were immunostained for paxillin, a focal adhesion marker, and visualized by immunofluorescence. As previously published, the *fak*^{-/-} MEFs exhibited large focal adhesions and cells re-expressing wild type FAK contained much smaller focal adhesions [38] (Figure 4A & B). The focal adhesions in cells expressing FAK mutants appeared to have an intermediate focal adhesion phenotype, i.e. paxillin-positive focal adhesions in cells expressing FAK mutants are smaller than those in *fak*^{-/-} cells, but larger than the focal adhesions in wild type FAK-expressing cells (Figure 4C,D,E). Thus, the mutants appeared partially defective in controlling focal adhesion size.

To confirm this qualitative observation, the average area of the focal adhesions in each of the cells was calculated (Figure 4F). As expected, cells re-expressing wild type FAK had significantly smaller focal adhesions ($0.85 \pm 0.27 \mu\text{m}$) than *fak*^{-/-} cells ($2.71 \pm 0.05 \mu\text{m}$). Cells re-expressing the R508A/K621A mutant exhibited significantly larger focal adhesions than cells re-expressing WT

FAK ($1.43 \pm 0.13 \mu\text{m}$, $P < 0.01$). Cells expressing the other two FAK mutants, R508A/R514A/K515A and K621A/K627A, also exhibited a larger average focal adhesion area than wild type re-expressing cells ($1.26 \pm 0.08 \mu\text{m}$ and $1.20 \pm 0.08 \mu\text{m}$, $P < 0.05$) (Figure 4). These size differences demonstrate that these mutants are partially defective for transmitting downstream signals that control a biological outcome regulated by the wild type FAK protein.

The role of FAK function in focal adhesion turnover is well established [39]. To assess the role of catalytic domain residues in regulation of focal adhesion dynamics, focal adhesion stability, assembly and disassembly rates were measured. As R508A/K621A exhibited the largest defect on focal adhesion size, this analysis was focused on this mutant. *fak*^{-/-} MEFs stably expressing wild type or R508A/K621A FAK were transfected with GFP-paxillin as a marker to monitor focal adhesion assembly and disassembly. Images were collected at one minute intervals over two hours. Images were analyzed through the Focal Adhesion Analysis Server (FAAS) [33]. Re-expression of wild type FAK in *fak*^{-/-} MEFs resulted in an increase in both the rate of assembly and disassembly resulting in a stability time of 18.55 minutes, compared with 30.07 minutes for *fak*^{-/-} MEFs (Table 1). Expression of R508A/K621A modestly increased the focal adhesion assembly and disassembly rates and the stability time of focal adhesions in these cells was 25.60 minutes. Thus, these basic residue mutations impair normal FAK function in regulating assembly and disassembly of focal adhesions.

Table 1.: Summary of Results from Focal Adhesion Analysis Server

Cell Type	Assembly Rate (min ⁻¹)	Disassembly Rate (min ⁻¹)	Stability Time (min)	n (FA)
WT FAK	$1.04 \times 10^{-1} \pm 0.035$	$1.55 \times 10^{-1} \pm 0.017$	18.55 ± 0.75	2064
FAK -/-	$1.01 \times 10^{-2} \pm 0.0065$	$9.88 \times 10^{-3} \pm 0.008$	30.07 ± 0.35	1285
R508A/K621A	$2.02 \times 10^{-2} \pm 0.0025$	$2.97 \times 10^{-2} \pm 0.0046$	25.60 ± 0.20	1775

Since FAK activity is required for rapid cell spreading on fibronectin the ability of the R508A/K621A mutant to promote cell spreading was also assessed [38]. *fak*^{-/-} MEFs and cells re-

expressing wild type FAK or R508A/K621A were transfected with GFP-paxillin to facilitate imaging. Cells were trypsinized, plated on fibronectin coated coverslips and spreading was monitored by time lapse video microscopy. Images were acquired every minute over a 2 hour period and spreading of individual cells was measured by calculating cell area at each individual time point (Figure 5). While MEFs re-expressing wild type FAK spread rapidly on fibronectin coated coverslips, *fak*^{-/-} cells took significantly longer to spread. Cells expressing R508A/K621A exhibited a dramatic defect in cell spreading, comparable to the spreading defect exhibited by the *fak*^{-/-} MEFs. These results support the hypothesis that the basic residues on the catalytic domain of FAK are required for biological function.

FAK Catalytic Domain Binds Phospholipids

Molecular dynamics simulations suggest that five basic residues in the catalytic domain of FAK can dock to PI(4,5)P₂ head groups in the membrane [28] (Figure 1). To experimentally test this hypothesis, the catalytic domain of FAK was expressed as a GST fusion protein and binding to PI(4,5)P₂ was measured. Two different experimental approaches were utilized to measure lipid binding, lipid vesicle co-sedimentation and fluorescence anisotropy. For lipid vesicle co-sedimentation studies, large unilamellar lipid vesicles composed of 60% phosphatidylcholine (PC) and 40% phosphatidylethanolamine (PE) were prepared as control vesicles and experimental vesicles containing 10% PI(4,5)P₂/50%PC/40%PE were made. GST or GST catalytic domain fusion proteins were incubated with lipid vesicles for 1 hour on ice prior to vesicle sedimentation at 100,000 x g for 1 hour at 4°C. The amounts of GST and fusion protein partitioning into the pellet (lipid bound) and supernatant (free) was determined by analyzing the two fractions by S.D.S-PAGE and Coomassie blue staining. In the absence of lipid vesicles, each protein was exclusively found in the supernatant (Figure 6A). In the presence of PC/PE vesicles, a fraction of the GST catalytic domain fusion protein co-sedimented with the vesicles (28.2%). A larger fraction of the GST catalytic

domain fusion protein (88.8%) co-sedimented with PI(4,5)P₂ containing vesicles (Figure 6). Under all conditions, the GST control remained in the supernatant fraction. To determine the role of the catalytic domain basic residues in vesicle binding, a mutant containing alanine substitutions for all 5 of these residues (Arg508, Arg514, Lys515, Lys621 and Lys627) was engineered (called RK5A) and analyzed for co-sedimentation with lipid vesicles. This GST mutant catalytic domain fusion protein was found in the supernatant in the absence of lipid vesicles. In the presence of PC/PE lipid vesicles, a fraction of the mutant fusion protein co-sedimented with the vesicles (20.6%). A similar fraction of the mutant fusion protein co-sedimented with PI(4,5)P₂-containing vesicles (27.6%) (Figure 6). These findings demonstrate that the FAK catalytic domain exhibits some binding to PC/PE vesicles, that binding is increased in the presence of PI(4,5)P₂, and that the basic residues on the catalytic domain play a role in binding to PI(4,5)P₂.

PI(4,5)P₂ binding was further validated using fluorescence anisotropy. GST and the GST catalytic domain of FAK were incubated with BODIPY-TMR labeled short acyl chain (C6) PI or PI(4,5)P₂ and anisotropy measured. Specific binding (ΔmP of GST-catalytic domain minus ΔmP of GST ΔmP of GST) was plotted against protein concentration and K_d for binding was calculated (K_d = 15.7 ± 6.5 μ M) (Figure 7). The wild type catalytic domain did not specifically bind PI and the RK5A mutant showed no specific binding to PI(4,5)P₂.

FAK Catalytic Domain Mutants Exhibiting Biological Defects are Defective for Phospholipid Binding.

To determine if the FAK mutants exhibiting defects in the control of biological processes also exhibited defects in phospholipid binding, the R508A/R514A/K515A, K621A/K627A, and R508A/K621A mutations were engineered into the GST-catalytic domain construct. Short acyl chain (C6) BODIPY labeled PI(4,5)P₂, was chosen to assess the ability of these mutants to bind phospholipids using fluorescence anisotropy. The R508A/R514A/K515A and K621A/K627A mutants each bound to the short acyl chain PI(4,5)P₂ (K_d = 16.4 ± 3.2 and 20.8 ± 2.7 μ M respectively) (Figure

7 C, D). In contrast, the R508A/K621A mutant was defective for PI(4,5)P₂ binding ($K_d > 200$ μ M)(Figure 7E). Thus, the mutants exhibiting modest biological defects were capable of binding PI(4,5)P₂, whereas the mutant with the severest defect in controlling biological responses was deficient in binding PI(4,5)P₂. These results support a role for these group II basic residues in the phosphatidylinositol phosphate binding activity of the catalytic domain and demonstrate that Arg508 and Lys621 are particularly important for phosphatidylinositol phosphate binding and regulating biological outcomes at the cellular level.

FAK Catalytic Domain Interacts with Multiple Phosphatidylinositol Phosphates

To test the specificity of the lipid interaction, the binding of wild type and RK5A GST-fusion proteins to phosphatidylinositol, PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ was analyzed. Purified GST catalytic domain fusion proteins were titrated into a solution of BODIPY-labeled phospholipids with C6-acyl chains, and anisotropy was measured. Phosphatidylinositol with C6-acyl chains and purified GST were used as controls. Differences in anisotropy of each BODIPY-labeled phospholipid at 100 and 200 μ M of each protein were compared by ANOVA and a Tukey's posttest (see Supplemental Table 1). The wild type catalytic domain showed significant binding to all phosphatidylinositol phosphates, but not PI, compare to the GST control and RK5A. Compared with the PI control, binding of PI(4,5)P₂ and PI(3,4,5)P₃ to the wild type catalytic domain was significant, while binding of PI(4)P approached, but did not reach significance. To calculate binding affinities, anisotropy was plotted against protein concentration and binding to GST was subtracted as background binding. The K_d of the wild type catalytic domain for PI(4,5)P₂ was calculated to be 12.9 ± 2.3 μ M (Figure 8B). Specific binding of wild type to PI(4)P and PI(3,4,5)P₃ resulted in K_d values of 11.7 ± 13.7 μ M and 16 ± 9.2 μ M respectively. To determine if the observed interaction with the short acyl chain PI(4,5)P₂ required the basic residues implicated in binding to PI(4,5)P₂-containing vesicles, the RK5A mutant was analyzed. This mutant exhibited binding similar to GST

with all phospholipids tested ($K_d > 200 \mu\text{M}$) (Figure 8C). These results demonstrate that the catalytic domain of FAK exhibits phosphatidylinositol phosphate binding, but that it does not discriminate between different phosphatidylinositol phosphate species.

Phospholipid Binding and Catalytic Activity

Although it was unlikely that substitution of basic residues on the surface of the catalytic domain with alanine residues would perturb enzymatic activity, catalytic activity of the wild type and RK5A mutant fusion proteins was measured. Four micrograms of protein were incubated in kinase reaction buffer containing poly(Glu,Tyr) as substrate and the generation of ADP was monitored using the ADP-Glo assay. GST alone showed no activity while the wild type GST catalytic domain demonstrated activity. The catalytic activity of the RK5A mutant was identical to the activity of the wild type fusion protein (Figure 9A). Therefore the mutations did not alter the enzyme activity of the catalytic domain and by inference did not perturb the structure of the domain.

An intriguing possibility is that lipid binding to the catalytic domain might directly modulate catalytic activity. To test this hypothesis, the GST catalytic domain fusion protein was incubated with PC/PE vesicles or PC/PE vesicles containing PI(4,5)P₂ in vesicle binding buffer, prior to performing the kinase assay. The catalytic domain exhibited the same activity in the presence of buffer only, PC/PE vesicles and PC/PE vesicles containing PI(4,5)P₂ (Figure 9B). These results demonstrate that association with lipid vesicles has no impact upon the enzymatic activity of the isolated catalytic domain.

Discussion

In addition to studies demonstrating PI(4,5)P₂ binding to the basic sequence on the F2 lobe of the FERM domain [24,25], a recent computational modeling study identified other potential PI(4,5)P₂ interaction sites on the catalytic domain [28]. We have validated PI(4,5)P₂ binding to the catalytic domain using both lipid sedimentation and fluorescence anisotropy approaches and mutation of the basic residues proposed to bind PI(4,5)P₂ impaired catalytic domain binding to PI(4,5)P₂. While identified basic residues did mediate this interaction, the catalytic domain of FAK also demonstrated binding to PI(4)P and PI(3,4,5)P₃. Thus the basic residues on the surface of the catalytic domain facilitate association with multiple phosphatidylinositol phosphates rather than a specific phosphorylated species.

Interestingly, mutation of a pair of spatially proximal basic residues, R508A/K621A, was sufficient to dramatically impair phosphatidylinositol phosphate binding. This mutant exhibited defects in tyrosine phosphorylation *in vivo* and in the control of biological events regulated by FAK. These results implicate these catalytic domain basic residues in FAK function *in vivo*, which can at least partially be attributed to defects in phospholipid binding. Mutation of other subsets of the catalytic domain basic residues had little impact on phosphatidylinositol phosphate binding in the fluorescence anisotropy assay and only partially impaired the control of FAK-dependent biological responses in cells. The R508A/R514A/K515A and K621A/K627A mutants could exhibit a more subtle phospholipid binding defect, not detected in the fluorescence anisotropy experiments, or a defect in association with a novel protein or ligand. . All five of these residues are conserved, in avian, murine and human FAK sequences. R508, K621 (the most important residues for biological function) and K627 are also conserved in *Drosophila melanogaster*. Interestingly, only one of these residues (K515) is conserved in the murine and human sequences of protein tyrosine kinase 2 (PYK2), a protein highly related in sequence to FAK [40]. In total, these observations support the

importance of these basic residues in the biochemical and biological function of FAK and suggest a function that is unique to FAK and not shared by Pyk2 [40] [41,42].

Phosphatidylinositol phosphate binding is a key component in many cellular signaling pathways and can serve to alter subcellular localization and/or enzymatic activity. A very well characterized example is the PI(3,4,5)P₃/Akt signal transduction pathway, where the generation of PI(3,4,5)P₃ at the membrane recruits protein kinase D and Akt to the membrane via their PH domains facilitating activation of Akt [43]. The mechanism of PH domain binding to phospholipids is well established. PH domains accommodate the headgroup of phosphatidylinositol phosphates, for example PI(3,4,5)P₃ and PI(4,5)P₂, in a basic pocket that recognizes charge and shape to provide phospholipid binding specificity [44,45]. A number of other lipid binding domains, e.g. FYVE and ENTH domains, similarly use a basic pocket to bind phosphoinositides [46,47]. In contrast, there are specific examples of phosphatidylinositol phosphate binding to basic residues exposed on the surface of a domain, rather than within a binding pocket. Examples of this binding mechanism include the FERM domain of FAK, which binds PI(4,5)P₂ through interactions with basic residues along a surface exposed α -helix [22], and the tail of vinculin, which interacts with PI(4,5)P₂ through interactions with basic residue side chains projecting from its surface [48]. Other modular domains exhibit phospholipid binding sites that do not contain a basic binding pocket. For example many phosphotyrosine binding (PTB) domains have phospholipid binding sites separate from their phosphotyrosine binding sites. The phospholipid binding motif in PTBs is not a conserved sequence but an electrostatic feature defined as a “basic crown” [49], and mutation of basic residues in these regions can abrogate lipid binding [50]. Additionally, a recent study showed that many Src homology 2 (SH2) domains bind plasma membrane lipids with a high affinity and that binding occurs through alternate cationic patches (ACPs) [51]. ACPs bind several membrane lipid molecules

simultaneously while leaving the phosphotyrosine binding site of the SH2 domain accessible for ligand binding.

The interaction of the FAK catalytic domain with the membrane via basic residues on the side of the catalytic domain is envisioned to leave the ATP and substrate binding sites accessible for catalytic activity. A few studies have address the structure of substrates in complex with kinase domains using peptides to mimic the interaction with the active site. In these structures, the substrates adopt an extended conformation [52–55]. Further, computational analysis reveals that phosphorylation sites reside in regions of proteins that are predicted to be disordered [56]. A main phosphorylation target of FAK catalytic activity is an autophosphorylation site, tyrosine 397 in the flexible linker between the FERM and catalytic domains [57]. In the autoinhibited conformation, the linker region containing tyrosine 397 binds to the FERM domain. Biochemical analysis reveals that in this conformation tyrosine 397 is a poor site for phosphorylation, compared with an extended conformation, suggesting that release of the linker from the FERM domain may be required for phosphorylation [19]. There is no structural information about the linker in any context other than the autoinhibited conformation, and predictions suggest that it is unstructured. A second substrate is the FAK binding protein, paxillin. The N-terminal half of paxillin, which is the location of its phosphorylation sites, is predicted to be intrinsically disordered. Since authentic FAK substrates are phosphorylated in disordered regions, we expect that these sites can access the FAK active site, even when adjacent to the membrane.

The phosphatidylinositol phosphate binding site on the catalytic domain, which lies on the side of the catalytic domain near the juncture of the small and large lobes of the kinase is at a site that could potentially regulate catalytic activity. The interaction of other ligands with the catalytic domain of kinases can alter activity. For example, binding of cyclin to cyclin-dependent kinases alters the small lobe of the catalytic domain to create a catalytically competent structure [58]. Small

ligands can also impact activity. For example, copper binding to the catalytic domain of MEK1 is required for enzymatic activity [59]. While these ligands bind on the opposite side of the kinase domain relative to the phosphatidylinositol phosphate binding site on the FAK catalytic domain, these precedents prompted an experiment to directly assess the impact of lipid binding upon catalytic activity. The results demonstrated that lipid binding has no effect upon the activity of the isolated FAK domain. These results are consistent with the conclusion drawn by Goni et al, that phosphatidylinositol phosphate binding does not directly regulate catalytic activity [25]. Since lipid binding does not directly regulate the activity of the isolated catalytic domain, the observed biochemical and biological defects associated with the phosphatidylinositol phosphate binding mutant reflects a role in regulating function only in the context of the full length FAK protein.

Goni, et al. report lipid binding experiments with a construct containing the FERM and catalytic domains of FAK, and the KAKTLRK mutant, which ablates the FERM domain basic patch, does not exhibit binding despite the presence of the basic residues in the catalytic domain [25]. The difference in results between studies could reflect differences in experimental conditions including vesicle and buffer composition. Conversely, the different results might demonstrate that PI(4,5)P₂ engagement of the FERM domain basic patch is a requirement for catalytic domain binding to phospholipids in the context of a larger construct, although such a requirement was not evident in molecular dynamics simulations. Interestingly, phospholipid binding studies using the construct containing the FERM domain and catalytic domain demonstrated cooperative binding [25]. This could reflect oligomerization of FAK [25]. However, the cooperativity of binding could also be explained by exposure of a second phosphatidylinositol phosphate binding site, i.e. the binding site in the catalytic domain. The critical mechanistic event relieving autoinhibition resulting in FAK activation remains to be conclusively demonstrated. PI(4,5)P₂ binding to the FERM domain is required, but insufficient for FAK activation [25]. It is possible that upon FERM domain binding

dissociation of the FERM and catalytic domains must occur prior to docking of the catalytic domain to phosphatidylinositol phosphates. It has not been conclusively established how the FERM domain/catalytic domain interface interactions are disrupted during FAK activation. An attractive model for activation is a mechanical mechanism, since FAK activation is sensitive to stiffness of the extracellular matrix outside the cell and requires the integrity of the cytoskeleton inside the cell [60,61]. A recent molecular dynamics simulation has demonstrated that sufficient force can disrupt the interaction between the FERM and catalytic domains without dissociating the FERM domain from the PI(4,5)P₂ and without disrupting the alpha helical structure of the FERM and catalytic domains [27]. Given this consideration, the catalytic domain interaction with phospholipids in the membrane may serve to stabilize FAK in an active conformation, rather than participate in the activation mechanism (Figure 10).

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Declarations of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Predicted PI(4,5)P₂ Binding Sites on the FAK Catalytic Domain. **A)** The surface view of the FERM (pink) and catalytic (blue) domains in the autoinhibited conformation is shown. The linker connecting the FERM and catalytic domains is colored gray. The F₁, F₂ and F₃ lobes of the FERM domain and the position of the PI(4,5)P₂ binding site are indicated. The basic residues in the large lobe of the catalytic domain that are proposed to bind PI(4,5)P₂ are colored cyan. **B)** Ribbon diagram of the catalytic domain of FAK demonstrating the location of the catalytic domain residues proposed to bind PI(4,5)P₂.

Fig. 2. Catalytic Domain Mutants of FAK Exhibit Defects in Phosphorylation. *fak*^{-/-} MEFs expressing wild type or mutant FAK proteins were analyzed to assess FAK tyrosine phosphorylation. **A)** Representative Western blots of cell lysates probed with the FAK 4.47 antibody (FAK) or the phospho-specific pTyr397, pTyr577, and pTyr861 antibodies are shown. **B-D)** Western blot quantification was performed using ImageJ. Phosphorylation levels were normalized to FAK expression levels. Mutant phosphorylation levels were compared to WT phosphorylation levels using a one-way ANOVA analysis with a Dunnett's post-test ($n = 6$ [pTyr397] or 10 [pTyr577 and pTyr581] experiments, $* = P < 0.05$).

Fig. 3. FAK Catalytic Domain Mutants Localize to Focal Adhesions. *fak*^{-/-} MEFs expressing wild type or mutant FAK were plated on fibronectin coated cover slips overnight, fixed, permeabilized and incubated with the anti-FAK 4.47 monoclonal antibody. Alexa Fluor 488-labeled anti-mouse secondary antibody was utilized for visualization. Scale bar = 10 μ m.

Fig. 4. FAK Catalytic Domain Mutants are Partially Defective in Controlling Focal Adhesion Size. **A-E)** *fak*^{-/-} MEFs expressing wild type or mutant FAK were plated on fibronectin coated cover slips, fixed, permeabilized and incubated with an anti-paxillin monoclonal antibody. Alexa Fluor 488-labeled anti-mouse secondary antibody was utilized for visualization. Scale bar = 10 μ m. **F.** The average

focal adhesion size in each cell line (+/- S.D.) was calculated using ImageJ ($n > 30$ cells for each group, >20 focal adhesions measured per cell, over 3 experiments). Areas were then compared using a one-way ANOVA with a Bonferroni Multiple Comparison post-test. The focal adhesion size in cells expressing each of the FAK proteins was significantly different from the focal adhesion size exhibited by *fak*^{-/-} cells ($P < 0.001$). The focal adhesion size in cells expressing the mutant proteins was also significantly different than the focal adhesion size in cells expressing wild type FAK (* $P < 0.05$, ** $P < 0.01$).

Fig. 5. The R508A/K621A Mutant is Defective at Promoting Cell Spreading. *fak*^{-/-} cells and *fak*^{-/-} cells expressing wild type FAK or the R508A/K621A mutant were transfected with GFP-paxillin as a fluorescent marker for time lapse imaging. Cells were trypsinized, taken into suspension and plated on fibronectin coated imaging dishes. After 5 minute incubation to allow for attachment image acquisition was initiated. Images were captured every minute for 120 minutes and cell diameters were determined using Nikon NIS Elements analysis software. The change in average cell area (+/- S.D.) for each cell type is plotted against time following initiation of imaging. ($n = 18-20$ cells over 4 experiments)

Fig. 6. FAK Catalytic Domain binds PI(4,5)P₂ Containing Lipid Vesicles. GST fusion proteins were incubated in buffer alone or with large unilamellar vesicles comprised of 60%PC/40%PE (PC/PE) or 50%PC/40%PE/10% PI(4,5)P₂ (PI(4,5)P₂). The samples were sedimented at 100,000 x g and the supernatant (S) and pellet (P) fractions analyzed by S.D.S-PAGE and Coomassie blue staining (panel A). The results of quantification of multiple experiments ($n=6$) is shown in panels B-D. The partitioning of GST (panel B), the wild catalytic domain of FAK (panel C) and the RK5A mutant catalytic domain (panel D) between supernatant (S) and pellet (P) fractions is shown. Gels were analyzed using ImageJ and the fraction of each protein in the supernatant and pellet (+/- S.D.) was plotted.

Fig 7: FAK Catalytic Domain Binds Short Acyl Chain PI(4,5)P₂. FAK catalytic domain GST fusion proteins were incubated with BODIPY labeled short acyl chain (C6) PI or PI(4,5)P₂ and fluorescence anisotropy measured as the change in mP. Specific binding was determined by subtraction of Δ mP for GST alone from Δ mP for the GST catalytic domain fusion protein. The average change in mP (\pm S.D.) as a function of protein concentration ($n = 3$ experiments) is shown. Specific binding of the wild type catalytic domain (A) and fusion proteins containing the RK5A (B), R508A/R514A/K515A (C), K621A/K627A (D) and R508A/K621A (D) mutations to PI(4,5)P₂ was determined. The dissociation constants for the interaction between the catalytic domain variants and PI(4,5)P₂ was calculated from the specific binding curves. Wild type $K_d = 15.7 \pm 6.5 \mu\text{M}$, RK5A $K_d > 200 \mu\text{M}$, R508A/R514A/K515A $K_d = 16.4 \pm 3.2 \mu\text{M}$, K621A/K627A $K_d = 20.8 \pm 2.7 \mu\text{M}$, R508A/K621A $K_d > 200 \mu\text{M}$.

Fig. 8. FAK Catalytic Domain Binds Multiple Phosphatidylinositol Phosphates. Fusion proteins were incubated with BODIPY labeled short acyl chain (C6) phospholipids and fluorescence anisotropy was measured. A) Δ mP for BODIPY labeled PI, PI(4)P, PI(4,5)P₂ and PI(3,4,5)P₃ in the presence of 100 μM GST, GST wild type catalytic domain and the RK5A mutant is shown (\pm S.D., $n = 3$ experiments). Specific binding (Δ mP of GST-FAK catalytic domain minus Δ mP of GST alone) of GST wild type catalytic domain (B) and the RK5A mutant (C) is plotted versus concentration. Specific binding to PI (circles), PI(4)P (squares), PI(4,5)P₂ (triangles) and PI (3,4,5)P₃ (inverted triangles are shown). The dissociation constant of the wild type catalytic domain for each phosphatidylinositol phosphate was calculated from the curves. PI $K_d > 200 \mu\text{M}$, PI(4)P $K_d = 11.7 \pm 13.7 \mu\text{M}$, PI(4,5)P₂ $K_d = 12.9 \pm 2.3 \mu\text{M}$, PI(3,4,5)P₃ $K_d = 16 \pm 9.2 \mu\text{M}$.

Fig 9. Lipid Binding Does Not Modulate Activity of Isolated FAK Catalytic Domain. The catalytic activity of GST fusion proteins was determined using poly(Glu, Tyr) as a substrate. The generation of ADP over time was measured using the ADP-GloTM assay protocol. A) The kinase activity of GST alone

(circles), the GST catalytic domain fusion protein (WT, squares) and the GST RK5A mutant fusion protein (triangles) were measured. The average concentration of ADP (+/- S.D.) generated in each reaction is plotted versus time ($n = 3$). Specific activities were calculated for both WT and RK5A proteins by taking the amount of product produced over a 5 minute period of time during the initial, linear portion of the reaction divided by time, divided by mass of fusion protein. This resulted in a specific activity of 1.025 ± 0.002 nmol/min/mg for GST-WT and 0.913 ± 0.004 nmol/min/mg for GST-RK5A. **B)** WT catalytic domain was pre-incubated in lipid sedimentation buffer (squares), buffer containing 60%PC/40%PE vesicles (triangles), or buffer containing 50%PC/40%PE/10%PI(4,5)P₂ vesicles (circles) for one hour prior to performing the kinase assay. The average ADP concentration (+/- S.D.) generated in each reaction is plotted versus time ($n = 3$). Specific activities were calculated for all conditions by taking the amount of product produced over a 5 minute period of time during the initial, linear portion of the reaction divided by time, divided by mass of fusion protein. This resulted in a specific activity of 0.460 ± 0.004 nmol/min/mg for buffer containing 60%PC/40%PE vesicles and 0.543 ± 0.006 nmol/min/mg for buffer containing 50%PC/40%PE/10%PI(4,5)P₂ vesicles.

Fig 10. Proposed Model of FAK activation. **A)** FAK in its autoinhibited conformation can dock to PI(4,5)P₂ (black) via the basic sequences in the FERM domain (black star in light gray domain). The interaction between the FERM and catalytic domains (dark gray) is disrupted via a poorly defined mechanism **(B)**, allowing the rotation of the catalytic domain and docking to membrane phospholipids via the basic sequences in the catalytic domain (white star) **(C)**.

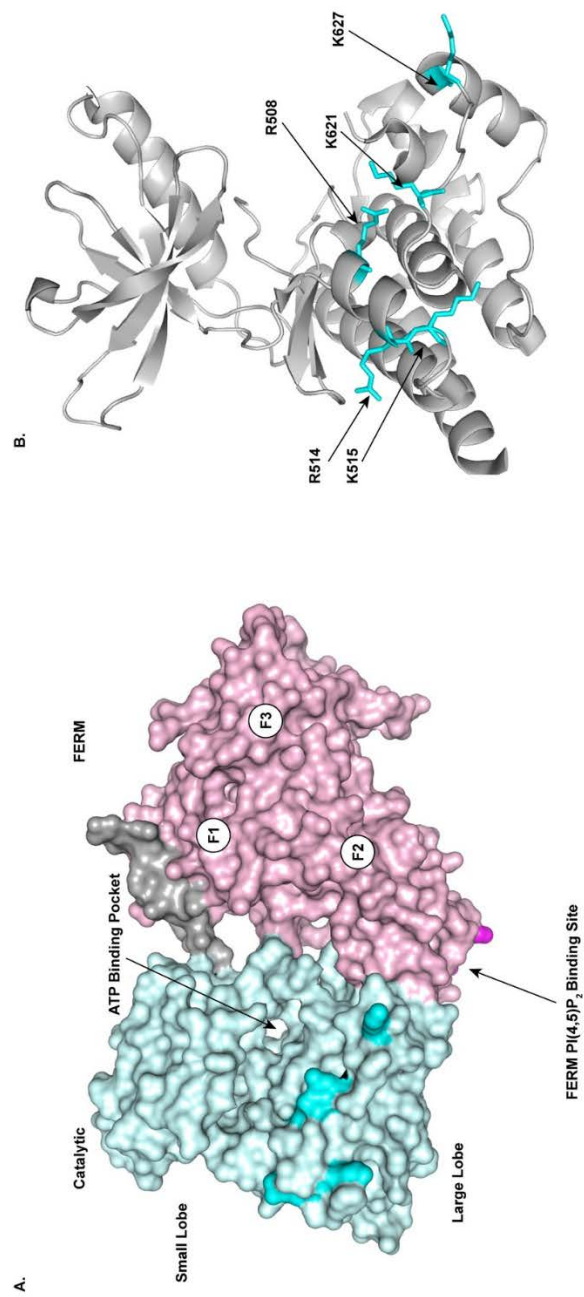
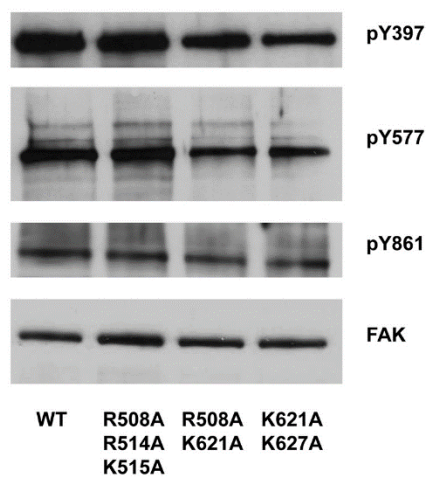
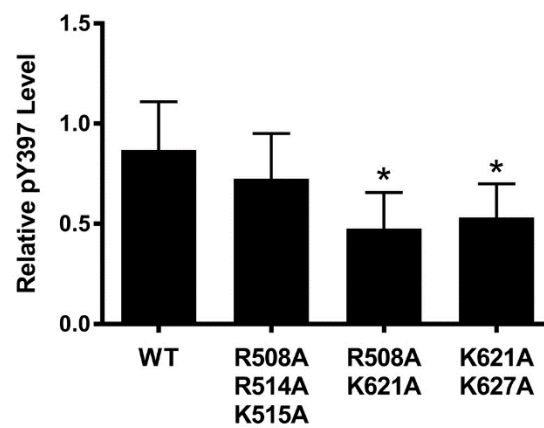


Figure 1

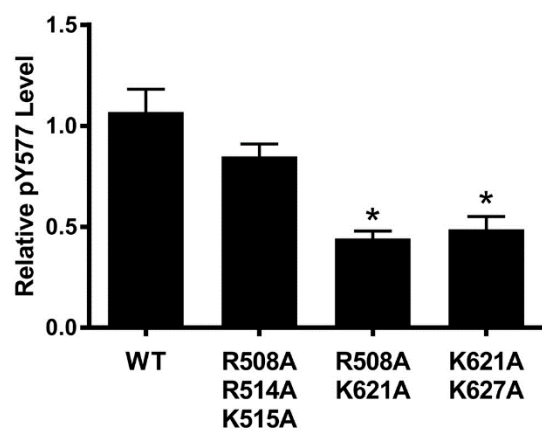
A.



B.



C.



D.

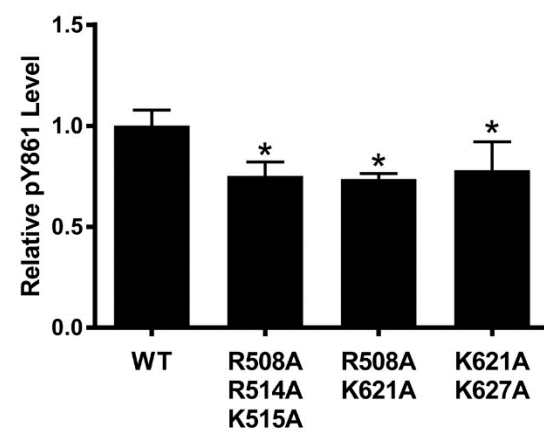


Figure 2

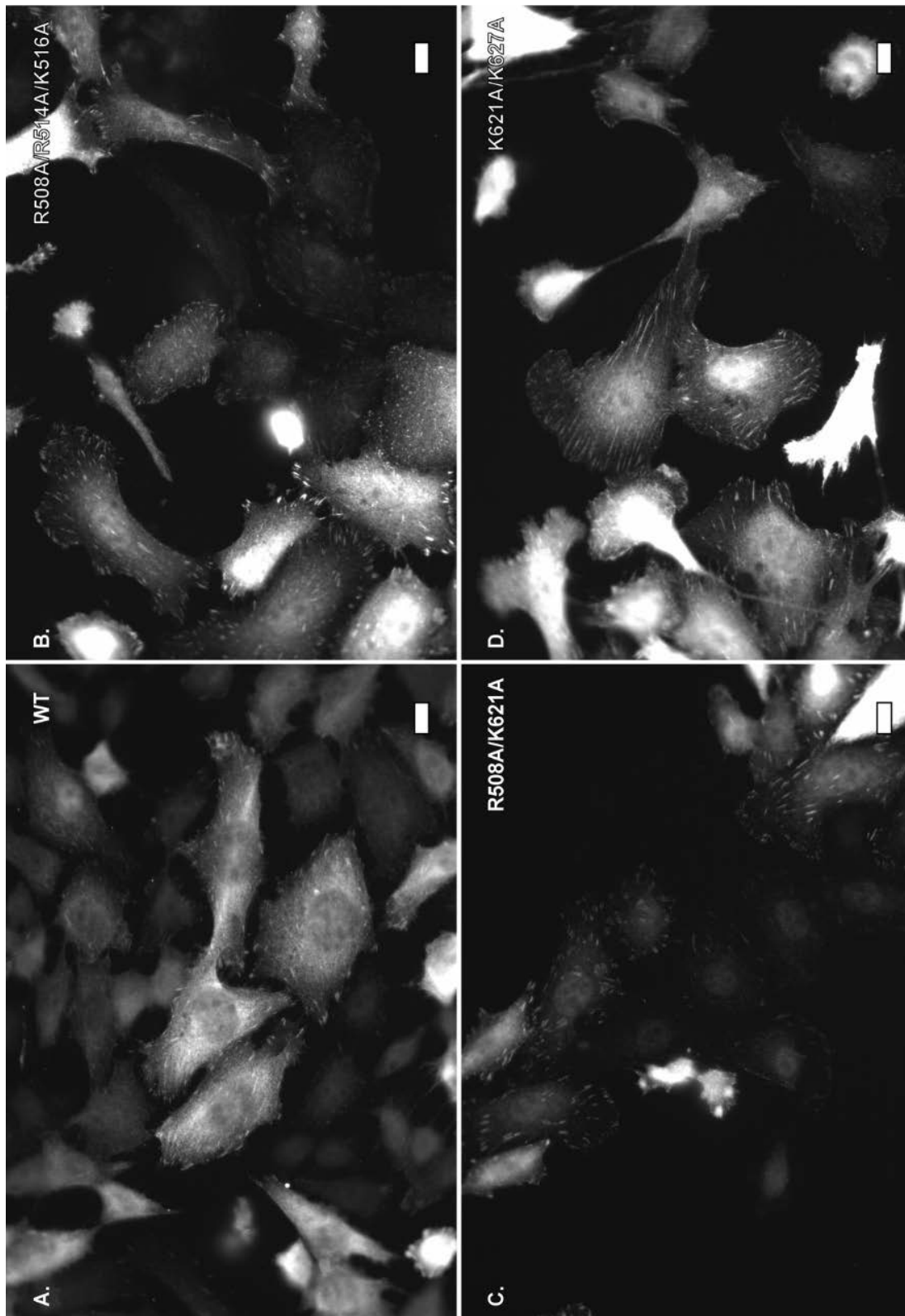


Figure 3

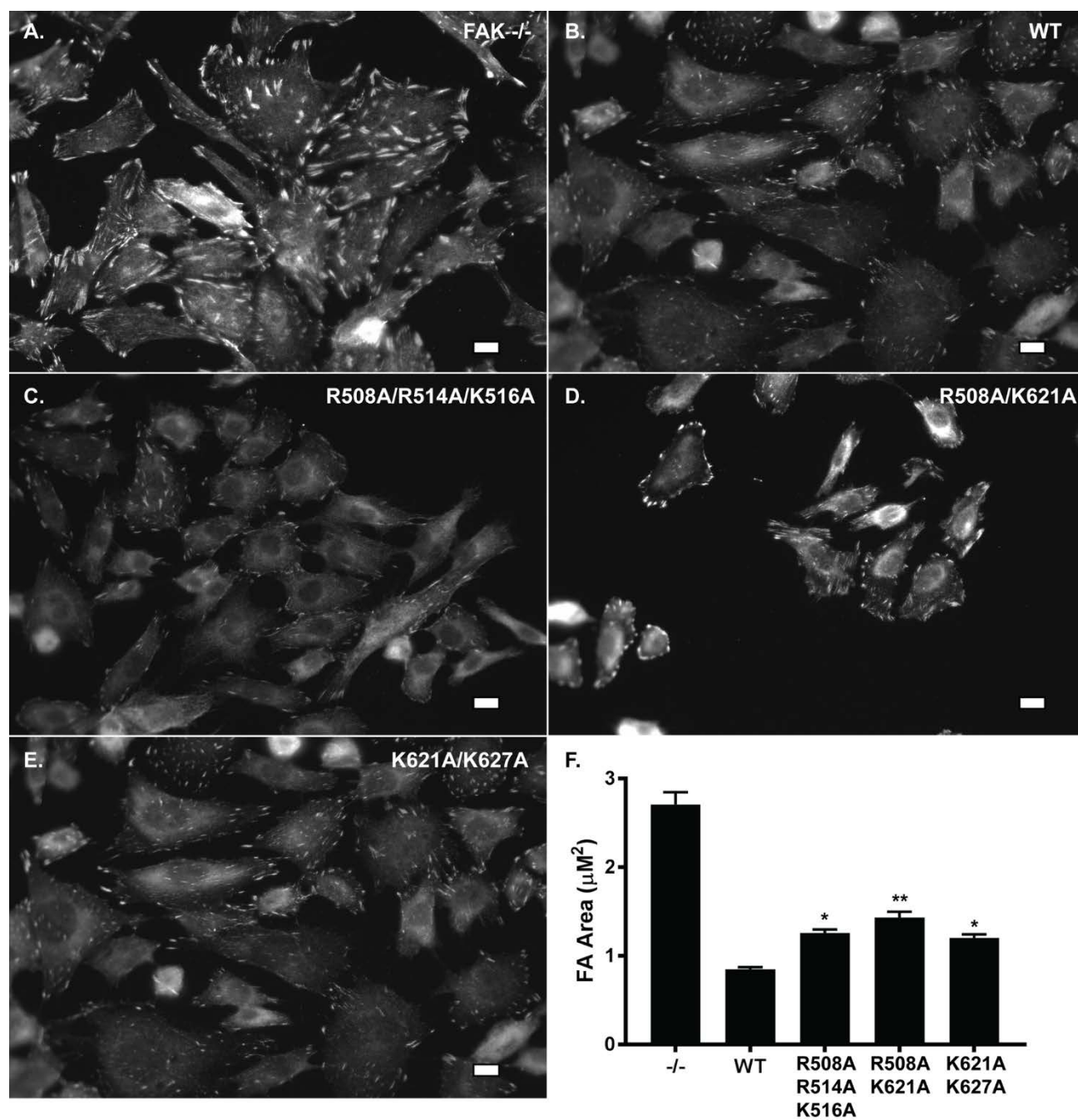


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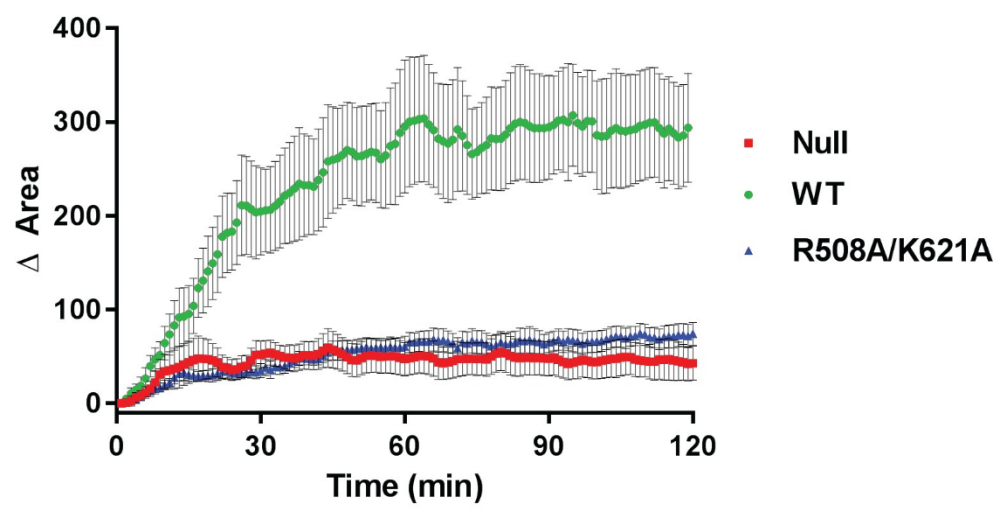


Figure 5

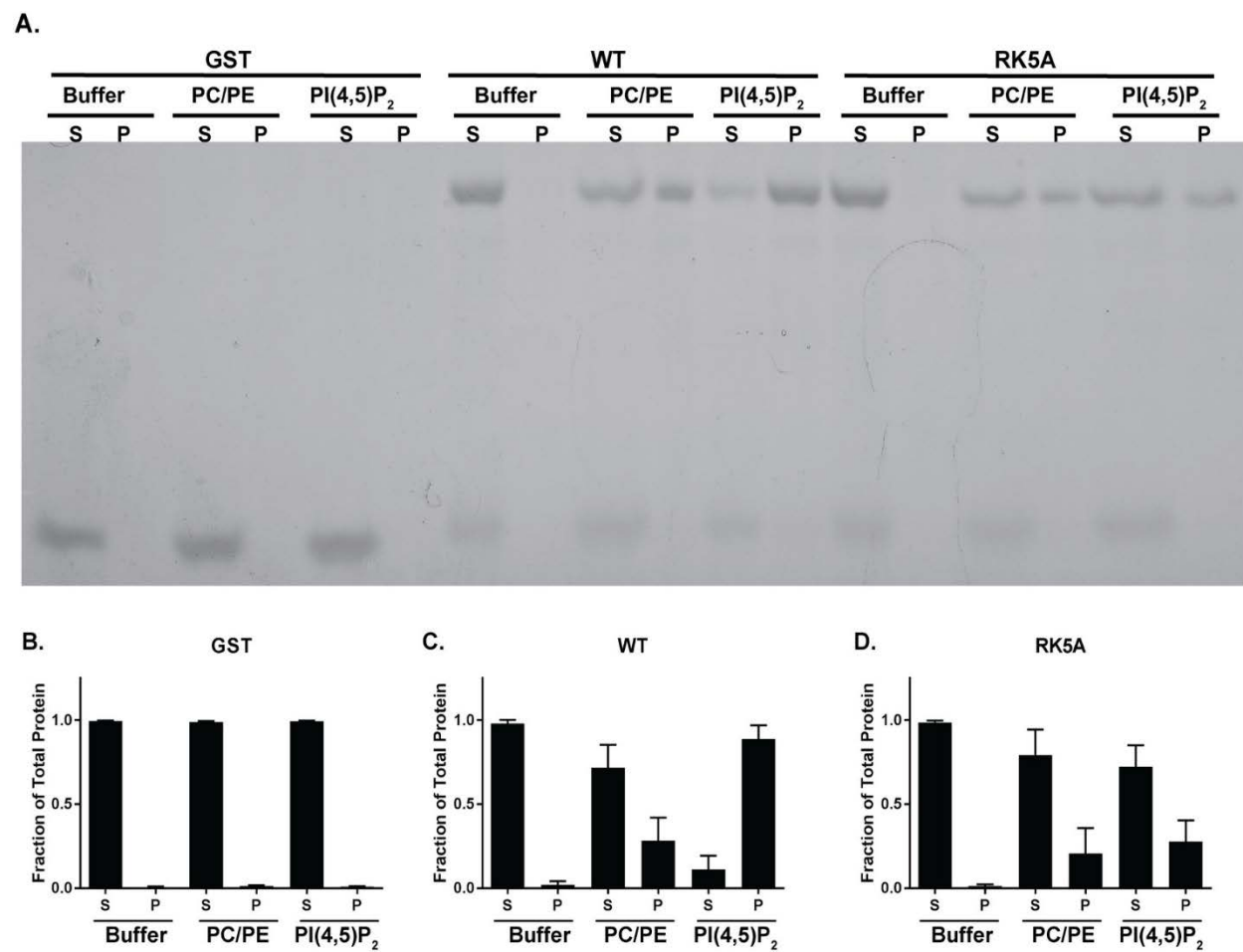


Figure 6

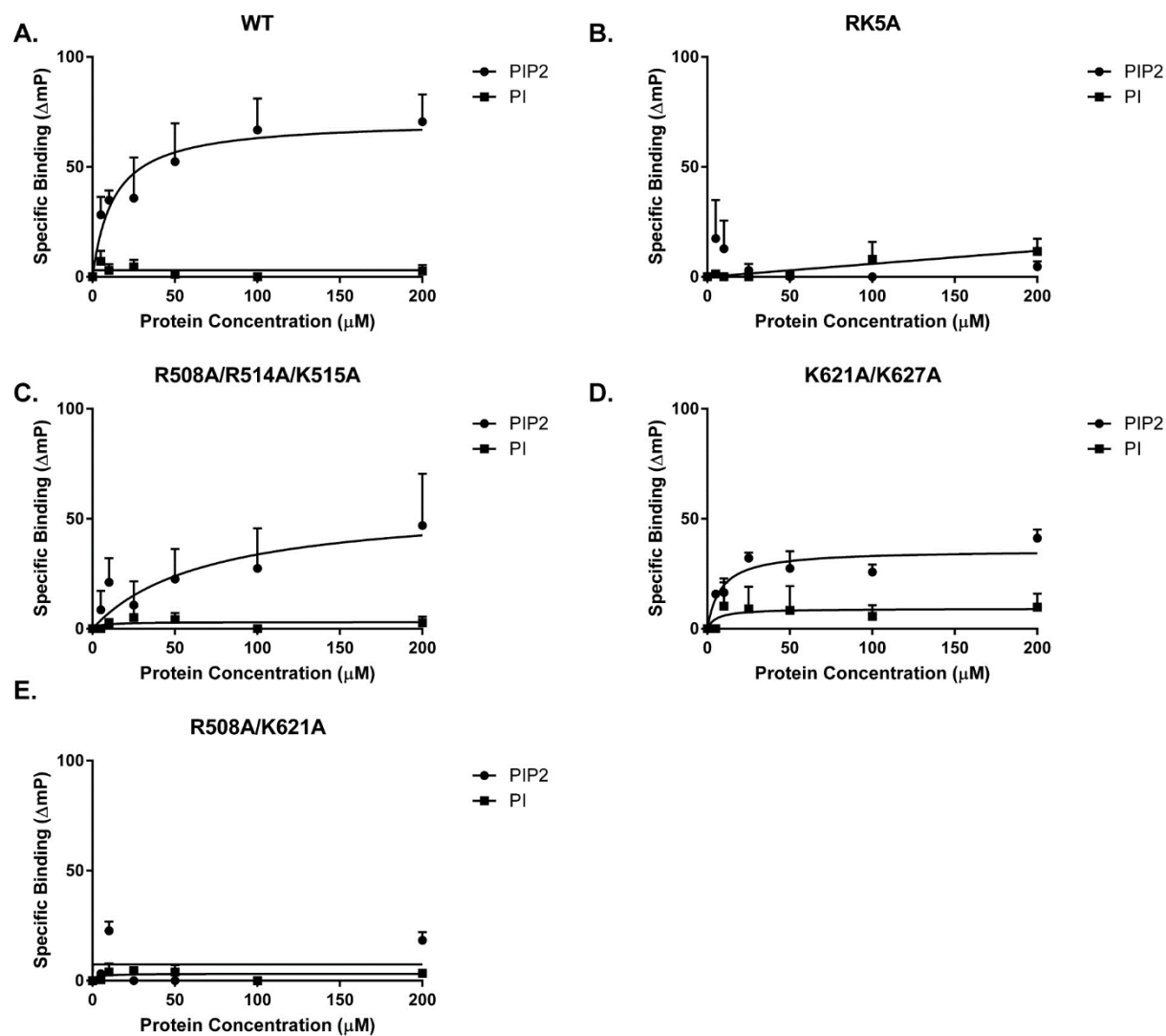
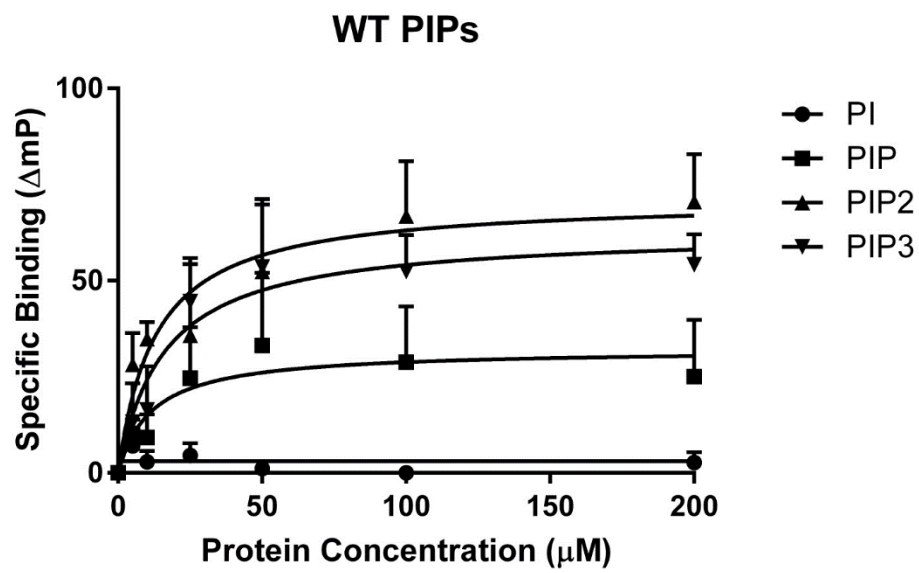


Figure 7

A.



B.

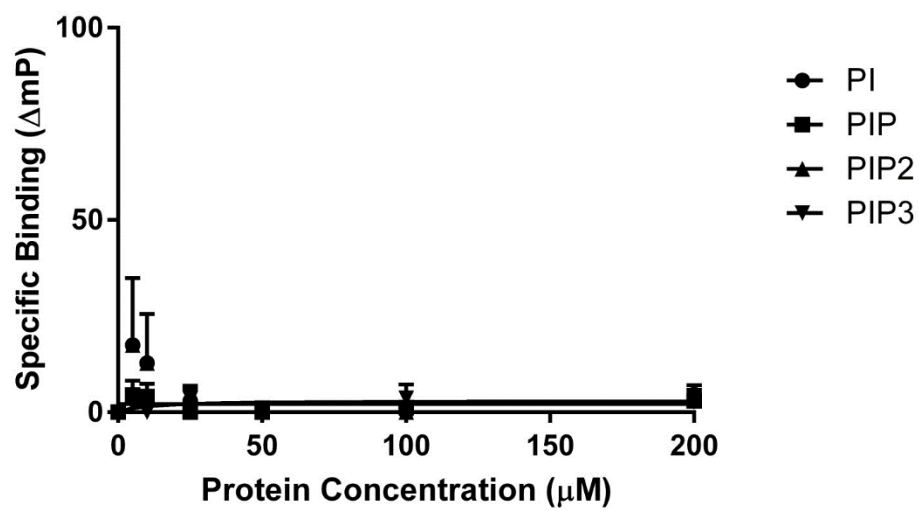


Figure 8

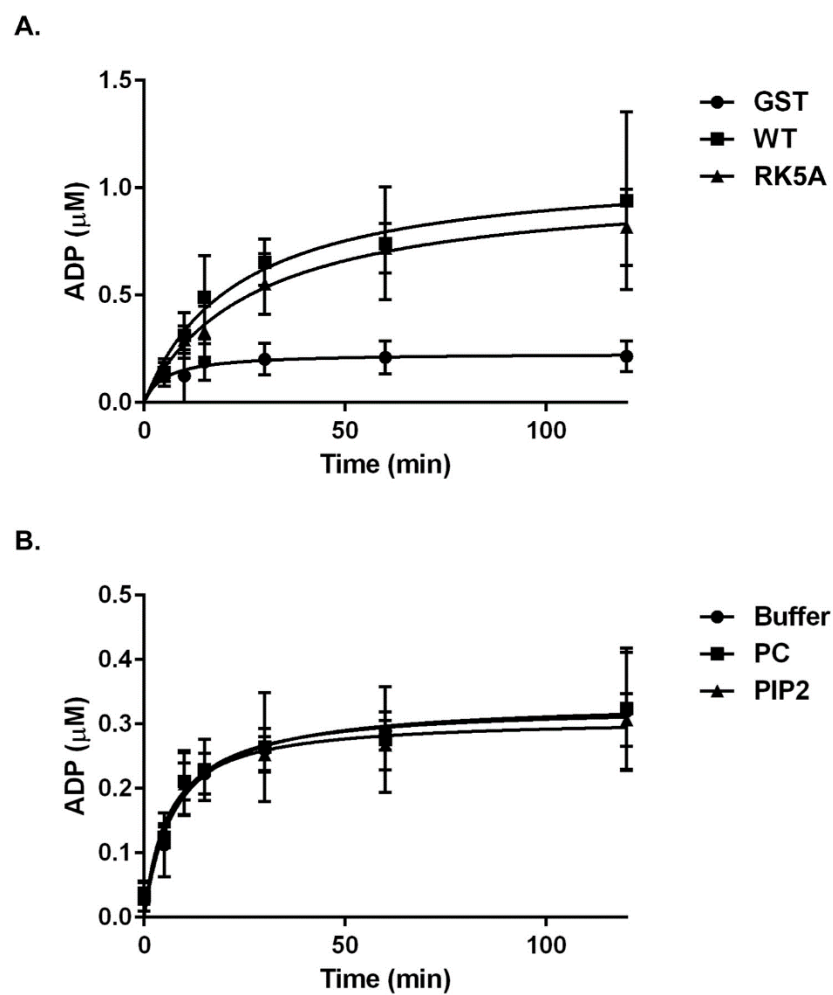


Figure 9

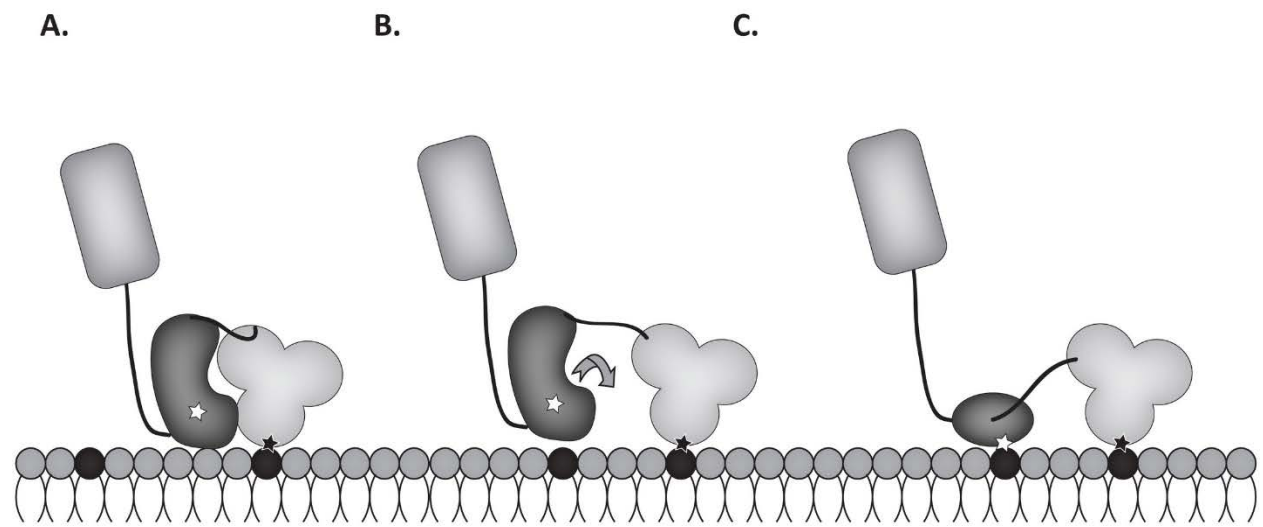


Figure 10

CHAPTER 4: Conclusions

Focal adhesion kinase is involved in many physiological processes that are essential to development of a multichambered heart and fully developed central nervous system. Additionally, with its role in cell migration it is necessary for wound healing (REF). Because of its roles in cell survival, proliferation, and motility FAK signaling is involved in many types of cancer (REF). As such, FAK activity needs to be tightly controlled. However, the mechanism for activation has yet to be fully determined.

FAK has three structured domains that contribute to the spatial control of activation. The C-terminal structured domain is the focal adhesion targeting (FAT) domain [1]. This domain is responsible for the localization to focal adhesions through interaction with paxillin [2]. Without localization to the focal adhesions FAK activation does not occur at any meaningful level. The N-terminal FERM regulates catalytic activity through an autoinhibitory conformation in which it folds over the active site of the kinase domain [3]. This conformation not only obscures the ATP and substrate binding sites but also sequesters the Y397 autophosphorylation site in the linker between the FERM and kinase domains through an antiparallel β -sheet formation. The F2 subdomain of the FERM domain contacts the C-lobe of the kinase domain, at this site F596 in the kinase domain inserts into a hydrophobic pocket in the FERM domain formed by Y180, M183, V196 and L197 [3]. Mutation of Y180 and M183 to alanine creates a constitutively active FAK, emphasizing the importance of these areas for maintenance of the autoinhibited conformation [4]. Additionally, a ridge of basic residues on the F2 subdomain, ²¹⁶KAKTLRK²²¹, binds to acidic ligands such as phosphotyrosine and PI(4,5)P₂ [4,5]. This allows for temporal control of FAK signaling as these interactions allow response to upstream activating signals. Through an unknown mechanism this ligand binding can trigger the release of the catalytic domain and exposure of Y397 and the ATP and substrate binding sites. This allows phosphorylation of Y397 which acts as the docking site for

Src. Src then phosphorylates the activation loop of the kinase, increasing catalytic efficiency, and tyrosines in the region between the kinase domain and the FAT domain. This series of activation steps leads to a fully active kinase with several binding sites, enabling a scaffolding function. The main question in this activation mechanism is how autoinhibition is released, as without the release of autoinhibition both catalytic and scaffolding functions cannot occur at their full efficiency. This dissertation set out to elucidate the molecular interactions that lead to a release of autoinhibition upon stimulation through investigation of key residues on the FERM and kinase domains.

Focal adhesion kinase is over expressed in many cancers and this overexpression is linked to poor prognosis. While the mutation rate for FAK is low, several mutations have been identified in the COSMIC database (<http://cancer.sanger.ac.uk/cosmic>). The initial stage of this investigation explored the effects of some of these single amino acid mutations found in patient tumor samples. While the mutation rate of FAK is low, there was a high potential that these mutations could lead to a dysregulated FAK molecule. Any effect caused by these mutations could provide insight into alternative mechanisms of activation. Mutations falling within the FERM domain, linker, and kinase domain were chosen as they were most likely to disrupt regulation. The first mutation, F147C, is located in the FERM domain, and could potentially disrupt the arrangement of the three FERM subdomains. The second, D402V, is located in the linker, this mutation has the potential to either disrupt the β sheet formed with the FERM domain or disrupt autophosphorylation at Y397. The final mutation from the COSMIC database analyzed was A590V. This mutation is within the kinase domain at the C-terminal end of the activation loop. This is part of the conserved APE sequence, a region rich in mutations in cancer associated kinases [6]. In biochemical assays measuring Y397 phosphorylation and catalytic activity F147C, and D402V were indistinguishable from wild type. In these assays the A590V mutant appeared catalytically dead. To assess the effect these mutants had on the autoinhibitory conformation they were analyzed utilizing a FRET based

biosensor. These results mimicked the biochemical data. F147C and D402V showed similar FRET levels as the wildtype biosensor and the A590V mutation lacked YFP emission signal indicating an improperly folded kinase domain. Overall these results work to further classify FAK in the context of cancer. There are two causes of aberrant kinase activation in cancer. The first are kinases that are mutated, like BRAF and TGFBR1 [6]. The second type, like HER2 and BRK, are kinases that are overexpressed [6,7]. The results of these mutation studies put FAK firmly in the overexpression category.

The second stage of investigation involved the rational design of mutants based on the crystal structures of the FERM domain and the FERM-kinase domains in the autoinhibited conformation. Special attention was given to the area where the FERM F2 subdomain and the C lobe of the kinase contact each other. Here F596 on the kinase domain fits into a hydrophobic pocket created by Y180, M183, V196 and L197 on the FERM F2 subdomain. Previous studies established that mutations of Y180, M183, and F596 can disrupt the formation of the autoinhibited structure [3,4]. On the apex of the F2 subdomain there is a ridge of basic residues, ²¹⁶KAKTLRK²²¹ [8]. This ridge is important for the maximal activation of FAK and binds phospholipids [4,9]. The alpha helix adjacent to the ²¹⁶KAKTLRK²²¹ helix sits directly above Y180 and M184. This helix contains a series of basic residues R184, K190, K191. A notable feature of these residues is their side chains and the peptide backbone adopt different conformations in the two crystal structures available. Based on this flexibility it was hypothesized that these residues may play a role in FAK activation, most likely through a change in conformation that would aid in the release of the kinase domain. Mutation of these residues in the FRET based biosensor did not lead to a significant change in FAK activation as measured by autophosphorylation. The mutants showed a slight change in basal level FRET, indicating a more open FAK, however this change was not statistically significant. Investigation of the change in FRET upon HGF stimulation showed no significant change in overall

FAK activity. However, these studies also showed a more variable response to HGF stimulation, indicating there might be some dysregulation of the control of conformation. These results suggest a minor role in controlling the autoinhibited conformation of FAK. While these residues are not key for response to upstream elements through triggering the conformational change they may be necessary for proper maintenance of the closed autoinhibited structure through contact with kinase domain residues.

The ²¹⁶KAKTLRK²²¹ binds to both phospholipids and phosphotyrosine, potentially triggering the release of autoinhibition and full FAK activation [4,5]. The RKK ridge while adjacent to the KAKTLRK ridge is partially buried in the autoinhibited conformation but may still be able to bind acidic ligands in concert with the KAKTLRK sequence. A recent study investigating the role of phospholipids in FAK activation showed an 2.5 fold increase in affinity for PI(4,5)P₂ when the interaction between the FERM and catalytic domains is destroyed by mutation of Tyr 180 and Met 183 to Alanine [10]. These mutations reduce the ability of FAK to hold the autoinhibited structure and increase the activity level of FAK [4]. A more open FAK would decrease the amount of time Arg 185, Lys 190, and Lys 191 are buried in the autoinhibited conformation. This could suggest that this basic ridge is responsible for the additional PI(4,5)P₂ binding observed. Additionally, two molecular dynamics simulations of FAK and PI(4,5)P₂ interactions identify Lys 190 and 191 as potential interactors with phospholipids [11,12]. The more recent of these two studies ran simulations with both the FERM and FERM-Kinase crystal structures. Simulations run with just the FERM domain suggest that residues buried in the autoinhibited conformation bind to the membrane once exposed, rotating the FERM domain and reducing the likelihood of returning to the autoinhibited structure [12]. Lys 190 and Lys 191 are among those implicated in these MD stimulations. Lipid sedimentation studies with the RKK mutated FERM domain showed a 10% defect in lipid binding. This decrease could account for the “nonspecific” binding observed when

similar studies are performed with the KAKTLRK mutant. Overall the results shown in this thesis and in other publications indicate that Arg 184, Lys 190, Lys 191 can interact with both the catalytic domain and the membrane, contributing to both the maintenance of the autoinhibited conformation and the maintenance of an open, catalytically active FAK. The results suggest that while these residues are not critical for either the autoinhibited or open conformations they may increase the stability in each.

The earlier molecular dynamics study show additional potential lipid binding sequences in the catalytic domain of FAK [11]. This study indicated that a series of basic residues, Arg 508, R514, K515, K621, and K627 were binding to the membrane through PI(4,5)P₂ [11]. A second *in silico* study confirmed the binding of Arg 508 and Lys 621 to PI(4,5)P₂ in both the autoinhibited and open conformations [12]. These residues are all located on one face of the catalytic domain, suggesting that this domain has a role in membrane binding in addition to the FERM domain. *In vitro* studies were performed to confirm these *in silico* observations [13]. These studies confirmed that basic residues on the side of the catalytic domain bind phospholipids. This binding was non-specific for phosphatidylinositol phosphorylation variant, but the domain did not bind to phosphatidylcholine or phosphatidylethanolamine. *In vitro* studies were followed up with *in vivo* studies to confirm biological significance [13]. In cells mutation of two basic residues R508 and K621 caused decreased FAK phosphorylation, an increase in focal adhesion area, and a defect in cell spreading. These *versus mundi* studies proved that catalytic residues are needed for lipid binding. Additionally they showed that mutation of only two residues, R508 and K621, can cause defects in cell spreading comparable to FAK knockout. Additionally, this second lipid binding site on the FERM-kinase structure could explain the avidity effect and lipid clustering observed in lipid binding studies performed with the two domains together [10]. The fact that there is an increase in lipid binding when comparing the FERM alone and FERM-kinase constructs but no difference between the FERM-kinase segment and

full length FAK suggest the catalytic domain is the segment of FAK outside of the FERM domain interacting with lipids.

The discovery of a second and, potentially, third membrane interaction site on the FERM-kinase autoinhibited structure modifies previous understandings of FAK activation. Both *in vivo* and *in silico* studies suggest that FAK is activated by mechanical force and acted as a mechanosensor [14,15]. However, none of this evidence conclusively proves that FAK itself is a mechanosensor and not simply downstream of other mechanosensory systems like integrins. Putting the FERM and kinase domain membrane binding into a mechanosensory context; it is currently unclear if the membrane binding functions actually trigger release of autoinhibition or whether they function to hold the FERM and catalytic domains apart and produce sustained signaling. In a model where the FAT domain is linked to the actin cytoskeleton through paxillin and the FERM domain binds to the membrane through ²¹⁶KAKTLRK²²², one can visualize force from cytoskeletal contraction pulling apart the autoinhibitory structure to trigger initial FAK signaling [14]. Upon a slight relaxation of the cytoskeleton the kinase domain could then rotate and bind the membrane, and create sustained signaling through preventing adoption of the autoinhibited conformation [13]. The two *in silico* studies of FERM and catalytic domain lipid binding suggest that the catalytic domain or other sites on the FERM domain bind more efficiently to PI(4,5)P₂ than the ²¹⁶KAKTLRK²²² sequence [11,12]. This would suggest a different mode of activation than the current mechanosensory model. An investigation of the binding strengths of both FERM, kinase, and FERM-kinase constructs for phospholipids to assess binding affinity should be conducted. Fluorescence anisotropy studies are a reliable method for determining quantifiable binding affinities and can yield data that would further inform these conflicting models of FAK activation.

Another intriguing aspect of all of the proposed lipid binding sites on FAK is that none of them create a binding pocket like other lipid binding domains (eg. FYVE or ENTH) [16,17]. However

the FERM domain lipid binding sites resemble the basic crowns found in some PTB domains that also bind lipid and the scattered charged residues on the catalytic domain are similar to the “alternate cationic patches” that enable some SH2 domains to bind the membrane [18]. So while not unprecedented, these lipid binding motifs are unexpected. The lack of a binding pocket for PI(4,5)P₂ likely explains the lack of specificity observed in lipid sedimentation studies [10,13]. Additionally, the ²¹⁶KAKTLRK²²² sequence is important for direct interactions with the phosphorylated tail of the met receptor [5]. This lack of specificity may be advantageous, allowing FAK to be activated by several different signaling pathways and differentially regulated in various cell types.

This expanded view of FAK phospholipid binding suggests a model in which these basic residues are necessary not for activation, but for maintaining the open conformation through extramolecular contacts. This model suggests an effect on the length of time the FAK signal is sustained by shifting the equilibrium to favor the open conformation when phosphoinositols are plentiful. In the context of dysregulated FAK signaling, especially in cancer, the signaling and regulation of lipid kinases is a vital component of aberrant FAK activity. The role of PI3K in cancer signaling is well established [19]. In the case of FAK the phosphatase PTEN could also be important for activation; mitigating its effect as a tumor suppressor by increasing levels of PI(4,5)P₂. Both this study and others show that while FAK has a generally nonspecific binding affinity towards PI phosphorylation variants, the FERM and catalytic domains bind better to PI(4,5)P₂ [4,10,13]. PI(4,5)P₂ is abundant in focal adhesions and integrin signaling specifically results in PIP5K γ recruitment [20,21]. Previous studies have concluded that PIP5K γ can contribute to cancer progression through activation of gelsolin and N-cadherin junctions, and its inhibition can reduce breast cancer metastasis [22,23]. PIP5 K γ is now further linked to cancer progression through FAK activation. The overexpression of FAK in many cancers is a poor prognostic indicator. This could

be due to a scenario in which you have more PI(4,5)P₂, to increase the sustained signaling of FAK and more FAK to respond, creating a cell in which both proliferation and motility are increased dramatically.

Because of its role in cancer progression FAK is an interesting target for therapeutics. Currently there are several FAK inhibitors in clinical trials. Most inhibitors designed for FAK are small molecule ATP-analogs that target the kinase domain [24–28]. In some cases these inhibitors while effective, do not prove useful in clinical trials due to off target effects on other kinase domain containing proteins [29,30]. Other approaches have been utilized in designing inhibitors for FAK. Compound 14 is designed to bind at Y397, thus preventing Src binding and full activation of FAK [31]. Another inhibitor targets the FAT domain and disrupts its interaction with paxillin [32]. The design of multiple inhibitors to target multiple sites on FAK will increase the odds of a successful therapeutic being developed [33]. The discovery of new areas on FAK that play a role in signaling through lipid binding increase the potential sites for inhibitor development. The residues making up the catalytic lipid binding site are not conserved in pyk2, FAK's closest relative [13]. This decreases the chance of the off target effects observed with ATP analog drugs. This research is the very beginning step towards potential new inhibitors, with better characterization of the FAK membrane interaction, the chances of a successful FAK inhibitor increase.

Overall, this work confirms that the catalytic domain of FAK binds to the membrane as proposed in an *in silico* model. Additionally, this interaction is necessary for a fully functioning FAK molecule and normal cell phenotype. Other portions of this dissertation suggest that the FERM residues K190 and K191 may have an impact on lipid binding as well. More work needs to be completed to fully characterize both these interactions. The effect of these residues on the length of time FAK stays in the active conformation needs to be assessed. Additionally the specificity of these sites could be better characterized. There is a potential for either of these sites to bind to

phosphotyrosines as well as phospholipids like $^{216}\text{KAKTLRK}^{221}$ does. This research will help to inform our understanding of FAK moving forward and may help establish a new model for signal persistence if not release of autoinhibition.

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EDUCATION

DOCTORATE OF BIOCHEMISTRY

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Advisor: Michael D. Schaller, Ph.D.

Thesis: The Role of Basic Residues in Focal Adhesion

Kinase Signaling and Activation

Defense Date: 2/15/17

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BACHELORS OF BIOLOGY

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TEACHING EXPERIENCE

Visiting Assistant Professor

Bucknell University

Biology 352: Cellular Biology

Biology 327: Molecular Biology

Biology 399: Undergraduate Research

Student: Jackie Ndem

Biomedical Engineering 431: Biomimetic Materials

Guest Lecturer on "Cell-Cell and Cell-Matrix Signaling"

August 2016 – Present

Lewisburg, Pennsylvania

Fall 2016

Teaching Experience While Graduate Research Assistant, WVU School of Medicine:

BIOC 531/705 General Biochemistry

Guest Lecturer on "Nucleotide Metabolism I & II"

Spring 2015

BIOC 339: Intro to Biochemistry

Guest Lecturer on "DNA Replication – Prokaryotes",
"Transcription", and "Gene Expression – Eukaryotes"

Fall 2014

BIOL 493: Molecular Biology of Cancer

Guest Lecturer on "Cancer Drug Development"

Spring 2014

BMS 706: Cellular Methods

Course Consultant: trained subsequent graduate students

Course Coordinator Team: designed curriculum

including lab activities, lectured in this course,
modified the course according to student feedback

Fall 2011 – Fall 2014

Other Teaching Experience

Tutor

WVU Intercollegiate Athletics

2007

Morgantown, West Virginia

RESEARCH EXPERIENCE

Graduate Research Assistant <i>West Virginia University College of Medicine</i> P.I. Michael D. Schaller	2010 – 2016 Morgantown, West Virginia
WVNano Initiative Grantee <i>NIOSH and West Virginia University College of Medicine</i> P.I.: Liying Wang and Tim Nurkiewicz	2009 – 2010 Morgantown, West Virginia
Regular Fellow <i>NIOSH</i> P.I.: Liying Wang and Robert Mercer	2008 – 2009 Morgantown, West Virginia
Crime Laboratory Assistant <i>Canton-Stark County Crime Laboratory</i> Laboratory Director: Robert Budgake	Summer 2006 Canton, Ohio
NASA-WV Space Consortium Grantee <i>WVU Department of Biology/NASA-WV</i> Work completed under Jeff D. Wells	2006 – 2007 Morgantown, West Virginia
Research Assistant <i>WVU Department of Biology</i> P.I.: Jeff D. Wells	2004 – 2007 Morgantown, West Virginia
Intern <i>Canton-Stark County Crime Laboratory</i> Laboratory Director: Robert Budgake	Summer 2004 Canton, Ohio

PUBLICATIONS

- 1) **Hall, J.E.**, Fu, W., Schaller, M.D., (2011) Focal Adhesion Kinase: Exploring FAK Structure to Gain Insight Into Function. *Int Rev Cell Mol Biol.* 2011; 288:185-225. PMID: 21482413
- 2) Fu, W., **Hall, J.E.**, Schaller, M.D. (2012) FAK Regulated Signaling Events in Cancer. *BioMolecular Concepts.* 2012; 3: 225–240
- 3) **Hall, J.E.** and Schaller, M.D. (2016) Phospholipid Binding to the FAK Catalytic Domain Impacts Function. *PLOS ONE* (Resubmitted after minor revisions)

PRESENTATIONS

Seminars

Focal Adhesion Kinase A Molecular Key to Heart Disease and Cancer – Susquehanna University February 17, 2017

Focal Adhesion Kinase A Molecular Key to Heart Disease and Cancer – Bucknell University March 23, 2017

Q is the Answer: Fatty Acid Synthesis in Hypoxic Conditions – Out of Area Seminar. December 10, 2013 WVU Health Sciences Center

Posters

- 1) **Hall, J.E.**, Feng, J., Mertz, B., Schaller, M.D. Novel Phospholipid Binding Sites on FAK. E.J. Van Liere Memorial Convocation & HSC Research Day, WVU Health Sciences Center. Morgantown, WV February 25- 2014
- 2) **Hall, J.E.**, Feng, J., Mertz, B., Schaller, M.D. Novel Phospholipid Binding Sites on FAK. Appalachian Regional Cell Conference, Charleston, WV October 26, 2013
- 3) **Hall, J.E.**, Mudry, R.M. & Schaller, M.D. Molecular Mechanisms Regulation FAK Activation. E.J. Van Liere Memorial Convocation & HSC Research Day, WVU Health Sciences Center. Morgantown, WV. February 22, 2012
- 4) **Hall, J.E.** & Schaller, M.D. Investigation of FAK Activation in Pathological Models. E.J. Van Liere Memorial Convocation & HSC Research Day, WVU Health Sciences Center. Morgantown, WV. March 11, 2011
- 5) **Hall, J.E.** & Schaller, M.D. Investigation of FAK Activation in Pathological Models. Science and Technology in Society: Effective Communication Strategies Poster Session, WVU Mountainlair. Morgantown, WV. April 5, 2011.

PROFESSIONAL DEVELOPMENT

New Faculty Seminar Series <i>Bucknell University</i> ; Lewisburg, PA This ongoing weekly series allows new faculty to learn teaching techniques, discuss problems and solutions, and assess progress throughout the semester.	Fall 2016
Course Design and Pedagogy Workshop for New Faculty <i>Bucknell University</i> ; Lewisburg, PA This course design workshop focused on introducing faculty to evidence-based pedagogical practices and assisted in course design and clear communication of objectives and expectations.	Fall 2016
Forensic Management Academy <i>Maryland State Police Crime Lab</i> ; Baltimore, MD This 5 day course taught skills to become more proactive and effective managers of laboratory staff and resources.	Spring 2016
WVU Teaching and Learning Commons Teaching Workshops These workshops train participants on pedagogy and diversity issues in higher education, provide mentored teaching experience, and educational assessment.	2013 – 2015

PROFESSIONAL SERVICE

Curriculum Subcommittee C This committee reviewed format and procedures for Individual Development Plans (IDPs) for graduate students. As a member of this committee I helped develop a plan for integrating IDPs more fully into the PhD curriculum and a guide for its use for advisers and students.	2015
Judge Cell Biology Poster Session, Appalachian Regional Cell Conference	2014

Seminar Coordinator Cell Biology Training Group Arranged for Dr. Ed Plow to come to WVU to tour facilities, speak to faculty and students about his research, and speak at a professional development luncheon for students.	2013
Seminar Coordinator Cell Biology Training Group Arranged for Dr. Peter Friedl to come to WVU to tour facilities, speak to faculty and students about his research, and speak at a professional development luncheon for students.	2012
Seminar Coordinator Cell Biology Training Group Arranged for Dr. Clare Waterman to come to WVU tour facilities, speak to faculty and students about her research, and speak at a professional development luncheon for students.	2011
Organizer Inaugural Appalachian Regional Cell Conference, now in its 5 th year.	2011

GRANTS SUBMITTED

- “Two-dimensional gel electrophoresis analysis of pollen from *S. asymmetriphyllum*” Pennsylvania Academy of Science Undergraduate Research Grant J. Ndem
Faculty Advisor: J. Hall
- “Molecular analysis of *S. asymmetriphyllum* pollen” BSA Undergraduate Student Research Awards. J. Ndem
Faculty Advisors: J. Hall & C. Martine

AWARDS AND HONORS

- | | |
|--|---------------|
| • First Place in Student Poster Session at Van Liere University Research Day | February 2014 |
| • Second Place in Cancer Cell Poster Session at Appalachian Regional Cell Conference | October 2013 |
| • Most Effective Communicator at Science and Technology in Society:
Effective Communication Strategies Poster Session | April 2011 |
| • WV Nano Grant | 2009-2010 |
| • The Sallie Lowther Norris Showalter Award for Excellence in Math and Science | Fall 2007 |
| • NASA- WV Space Grant Recipient | 2006-2007 |
| • Summer Undergraduate Research Experience (SURE) Grantee | Summer 2005 |
| • Blue and Gold Scholarship Recipient | 2003-2007 |

PROFESSIONAL AFFILIATIONS

Pennsylvania Academy of Science (PAS)
American Society of Cell Biology (ASCB)
Association of Women in Science (AWIS)
American Association for the Advancement of Science (AAAS)